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UNIVERSITY OF ALBERTA

EFFECT OF ENDOTOXIN ALTERATION ON *BORDETELLAE* OUTER MEMBRANE FUNCTION

BY

MARIE LAURINA TURCOTTE ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

EDMONTON, ALBERTA

SPRING, 1996

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ISBN 0-612-10646-2

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DEGREE:	Doctor of Philosophy
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THE UNIVERSITY OF ALBERTA

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This thesis is dedicated to my other "half" (a mathematical understatement); my family (my rock), who pushed, pulled and at times forcibly hauled my tookus through this degree:

Pops and Mom (John Jr. and Laurette Turcotte to the taxman).

My warped Siblings (with embarrassing middle names in print for posterity):

David Ralph Joseph, Suzan Agnes Mary, Rosemarie (who got off easy),

Bernard Dean John (who made up for Rose) and Barbara-Anne Tina.

My "non-genetic" Siblings (who have cannily concealed their middle names over the years): Lori, Mike, Shawn and Lisa.

My Monsters - my sunshine, (my Saturday night dates):

Lara Danielle, Kira Elyse, Melissa Anne, Dylan Thomas, Kory Shawn-John, Shannon Laur, Sydney Laurina and Hannah - all unstoppable forces with immovable attitude.

Thanks to my constructed family - the friends who have been there for me when "there" was not a perky, cha-cha place to be: *The Freaks*- Carol Ewanowich, Debby (Robinson) Hemmerling, Liane Kim, Christine Szymanski (yes, you are), Halah Alhussaini, Vicki Carter, and Kate Agopsowicz, *The "Norms"*- Peter and Elizabeth Newnham, Harpreet Punia, Marj Kelm, Sharee Kuny, Trevor Stenson, Rosa Vanmaele... and my fellow serfs: Margo Diado, Alma Hall, Vicki Jajczay, Heather Mitchell, Mary Olsen, San Vinh, Ken Whelen, Imtiaz Zainul and anyone that my 2 O'clock in the morning brain fog has momentarily zapped.

ABSTRACT

Much of what characterizes the processes of infection and immunity is determined at the bacterial surface. Precise molecular organization is very important to the structural and functional properties of the Gram-negative outer membrane. Alterations in endotoxin structure have been proven to affect almost every structural aspect and functional component of the outer membrane. Previous studies, centred on enteric species, indicated that the required alterations are extremely structurally specific and vary from species to species.

The *Bordetellae* are multifactorial pathogens which antigenically modulate from virulent to avirulent form in response to environmental stimuli. These switches induce drastic changes in the outer membrane yet little has been determined of the functional role or structural associations of endotoxin in either the outer membrane or bacterial pathogenicity. I undertook a number of studies in an attempt to clarify the significance of *Bordetellae* endotoxin to the function and nature of the bacterial cell.

My investigations examined the role of the antigenic, interactive core and O-unit moieties of the endotoxin molecule, in *Bordetella* outer membrane structure and function. To explore the in vivo relevance of *Bordetellae* endotoxin a series of *B. pertussis, B. bronchiseptica* and *B. parapertussis* mutants with altered endotoxin were created and characterized. The alterations resulted in disruption of outer membrane structure and function of all three species as was shown by differences in mutant reactivity to strain specific monoclonal antibodies and notable alterations in mutant sensitivity to human sera and hydrophobic as well as

hydrophilic antibiotics. Both qualitative and quantitative differences were noted in the extent of outer membrane disruption due to human sera as well as various antibiotics when endotoxin mutants of the three *Bordetella* species were compared.

The collective data of these investigations establish the importance of *Bordetella* endotoxin in the structure and function of the outer membrane. In addition they provide a foundation upon which future studies into the nature and clinical relevance of the association between endotoxin and other outer membrane components may be investigated.

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CHAPTER I

Introduction

A. GENERAL CHARACTERISTICS OF THE *BORDETELLAE*

The *Bordetellae*, a genus of Gram-negative pathogens, are a common cause of upper respiratory tract infections in a number of hosts. The genus consists of four species; *Bordetella pertussis* and *Bordetella parapertussis* are common human pathogens, *Bordetella bronchiseptica* is found rarely in humans but is a common animal pathogen and *Bordetella avium*, a bird pathogen. The differences between the species are notable and are discussed further below.

The numerous unifying similarities are equally worthy of scientific investigation. With the exception of *B. avium*, the *Bordetellae* share a significant amount of genetic and structural homology (8,80,83,129). *B. avium*, a recent addition to the genus, has far less resemblance genetically or structurally to the rest of the *Bordetellae* and substantial doubt has been raised as to its taxonomic placement. This study centered on the three species of *Bordetella* with a history of human infection and well documented commonalties.

B. pertussis, B. parapertussis, and *B. bronchiseptica* are piliated aerobic pathogens which require nicotinamide for growth, oxidize amino acids and have DNA with a high G/C content (66 - 70%) (146,223). DNA hybridization and multilocus enzyme electrophoresis studies have indicated a high degree of homology among the three species (80,83,129). Indeed, the argument has been raised that there is not enough genetic distinction to justify designation of the three as separate species. The following section provides a brief overview of the numerous common factors and mechanisms which have been implicated in *Bordetellae* pathogenicity.

1. Phase Variation and Antigenic Modulation

The homology between the three mammalian *Bordetella* species extends to the large number of virulence-associated toxins and factors possessed by the *Bordetellae*. Virulent organisms can be differentiated from avirulent organisms by differences in colony morphology, antigenic reactivity and pathogenicity (42,143,146,147). While all three of the *Bordetella* species in this study exhibit characteristic shifts from virulence to avirulence (and back under certain circumstances) most studies to date have centered on the specifics of *B. pertussis* conversion.

Leslie and Gardner first characterized the ability of *B. pertussis* to change in this manner in 1931 (95). They established a typing system in which four phases of *B. pertussis* were possible. Phase I organisms produce hemolytic, virulent colonies. Phase II bacteria produce non-hemolytic, virulent colonies. Phase III and IV organisms result in non-hemolytic, avirulent phenotypes differentiated from each other by the ability of Phase IV bacteria to grow on unsupplemented nutrient agar(95,143). Preston later proposed a different typing scheme as did Kasuga and Nakase(77,78,147). Regardless of the steps involved, agreement has been reached as to the existence of two separate phenomena by which the conversion from virulent to avirulent phenotype routinely occurs. The first, termed antigenic modulation, denotes the reversible shift of an entire population under certain environmental conditions. The second such phenomenon is called phase variation and refers to the largely irreversible switch of a very small population subsection (approximately 1 in 10^6 organisms) due to mutation of a specific genetic locus. This trans-acting, regulatory locus [deemed the *bvg* (*Bordetella* virulence gene) locus], is responsible for antigenic modulation as well as phase variation (8,34,191,211).

Several groups, notably those of Falkow and Rappuoli, have determined *bvg* to encode a two component coordinate regulation system which regulates the production of a number of unlinked virulence determinants in response to environmental stimuli. This system consists of the BvgA and BvgS proteins which are encoded by the *bvgA* and *bvgS* genes, respectively (115,189,190,191). The *bvg* locus is a common genetic element in all three *Bordetella* species (8,144,191). The loci of all three species are functionally homologous. As well, in each of the three species, regions of the locus with putative kinase and DNA-binding activities are conserved. Possible sensory regions are better conserved between *B. parapertussis* and *B. bronchiseptica* (8).

The system works as follows. Under non-modulating conditions (defined as 37°C, low $MgSO_4$, low nicotinic acid present) the BvgS protein (the sensory portion of the locus) is produced and activates the BvgA protein (the regulator component) through site-specific phosphorylation of a highly conserved aspartate residue (187,191,199). Phosphorylated BvgA then initiates production of bvg-regulated molecules through trans-activation of sensitive promoters. Promoter-activation can be direct as has been found to be the case in autoregulation of BvgA and activation of the locus which encodes filamentous hemmaglutinin (167,191). More complex, trans-acting factors are involved in the activation of pertussis toxin and adenylate cyclase production (175,191).

It was Lacey who first induced the population shift from "X" (xanthic) mode to "C" (cyanic) mode which was later termed antigenic modulation (87). This shift of an entire population from virulent to avirulent (and vice versa) is due to certain environmental stimuli. Low temperature (25°C-28°C), and certain salts (50mM $MgSO_4$, 10mM nicotinic acid) switch off the BvgS protein which in turn inactivates the BvgA protein. The lack of promoter trans-stimulation causes the down-regulation of the vir-activated genes (*vags*) and the up-regulation of vir-repressed genes (*vrgs*) to produce vir-repressed gene products(8,33,34,191). Removing the external stimuli results in the reciprocal activity (up-regulation of *vags* and down-regulation of *vrgs*) (2,3,13).

Many bacteria have such coordinate regulation systems as a means of adapting to environmental changes (115). It is often advantageous for a pathogen to vary its surface components as they are the stimulus presented to a host's immune system. In many pathogens these changes translate to the difference between the extracellular and intracellular environments. Although all three species of *Bordetella* have been found to be invasive in several transformed and primary cell lines, the role, if any, of antigenic modulation in invasion and bacterial persistence in the host is still a matter of study (45,46,177).

Phase variation refers to a second phenomena where, upon repeated in vitro subculture, a small portion of a given population (approximately 1 in 10^3 organisms) undergoes the shift from virulence to avirulence (144,187,188,191,211). This is notably higher than would be expected from spontaneous mutation of the *bvg* locus (approximately 1 in 10^6). Phase variants are far less likely to revert to virulence than antigenically modulated cells (187,188,191,211). The source of phase variation in *B. pertussis* is the frameshift mutation of a single G in a string of 6 Gs at the end of *bvg*S (188). This differs slightly from *B. bronchiseptica* where phase variation is caused by small spontaneous deletions which can occur throughout the *bvg* locus (118). This variance may explain the increased rate of phase variation observed in *B. bronchiseptica* as compared to *B pertussis*.

The molecules which are regulated by the *bvg* locus vary widely in both form and function. Most, however, are associated with Bordetella virulence and pathogenicity (191,209,211,212,213).

2. Virulence Factors

Bvg Regulated Molecules

i) Pertussis Toxin - Pertussis toxin (PT) is perhaps the most intensely scrutinized product of *B. pertussis*. Historically, it has been known by many names mirroring its ability to elicit a wide range of local and systemic reactions. Since its initial isolation it has been known as histamine sensitizing factor (HSF), islet-activating factor (IAF) as well as lymphocytosis-promoting factor (LPF) (200). All these names stem from the clinical manifestations attributed to the ability of pertussis toxin to catalyze the ADP-ribosylation of GTP-binding proteins ($G_{inhibitor}$ protein) of many cell types (52). It is the cell type which dictates whether the principal consequence of the toxin is an increase in adenylate cyclase activity, decrease in phospholipase activation, decrease in activation of ion channels and Ca^{+} mobilization or some combination of these events (52,125,127). Systemic and local effects attributed to PT include anaphylaxis, and induction of leukocytosis (200).

Pertussis toxin is a 105 kDa protein with a classic two component (A-B) toxin structure composed of 5 protein subunits, S1-S5. The enzymatic ("A") moiety is composed of a single polypeptide, the S1 subunit. The binding ("B") component is a pentameric ring formed by the S2, S3, S5 and two S4 subunits. The oligomeric protein is assembled in the periplasm before export to the cell surface (75,127). Pertussis toxin is encoded by the *ptx* operon which, although not physically linked to the *bvg* locus, is nonetheless under its control through the trans-activation of its promoter by BvgA (66).

In addition to its systemic and local effects, pertussis toxin is thought to play a major role in adherence of the organism to a host cell and subsequent invasion (109,196,209,210,212,214). Although analogous *ptx* genes are present in all three

Bordetella species, mutations within the *ptx* promoter regions in both *B. parapertussis* and *B. bronchiseptica* result in a lack of expression of the *ptx* gene and a subsequent lack of the pertussis toxin protein (168).

ii) Adenylate Cyclase Toxin/Hemolysin - Adenylate cyclase toxin (cya) and hemolysin (hly) are functionally independent activities expressed by different regions of the 43kDa multifunctional protein, cyclolysin, which is encoded by the *cyaA* gene in the *cya* operon. An intracellular adenylate cyclase toxin which lacks a signal sequence is also encoded by this operon. The *cya* operon consists of four contiguously arranged genes, *cyaA, cyaB, cyaD,* and *cyaE. CyaB, cyaD* and *cyaE* are located immediately downstream of *cyaA* and are required for the secretion of the cyclolysin protein(62,63,73,169).

Extracellular adenylate cyclase toxin, once secreted by *B. pertussis,* binds and enters eukaryotic cells upregulating production and accumulation of cAMP in the presence of host calmodulin. The toxin encompasses two domains on the cyclolysin, a 25kDa N-terminal catalytic site and a 13kDa C-terminal domain which serves as the principal calmodulin binding site (62,124). Although the *cyaA* gene is able to independently mediate binding of the toxin to target cells, post translational modification by the product of an accessory gene, *cyaC,* is required for transmembrane delivery of the catalytic domain of the toxin and the intracellular accumulation of Ca^{++} (12). Once inside a target cell, the 216 kDa holotoxin rapidly catalyzes the conversion of ATP to cAMP in the presence of endogenous calmodulin. This increase in intracellular cAMP levels may merely impair normal cell functions or, if great enough, have pronounced cytotoxic effects (198). Recent data have also implicated extracellular adenylate cyclase toxin with the production of ion-permeable, cation-selective pores in phospholipid bilayers (15,192).

Although the properties of adenylate cyclase toxin are formidable in themselves, it has been established that in order to initiate an infection *Bordetellae* must possess a cyclolysin which expresses hemolytic as well as cya properties (67). The hemolysin activity of cyclolysin is thought to play a critical role in the entrance of adenylate cyclase toxin into eukaryotic cells. Mutants in either cya or hly are much less virulent (67,81,210,213,214). Thus, both hemolytic and toxic activities are considered important in slowing bacterial clearance and ensuring persistence of *B. pertussis* in the host (112).

iii) Filamentous Hemagglutinin/Fimbriae - Filamentous hemagglutinin (FHA) is a 220 kDa rod-like protein which is cleaved from a 370 kDa precursor. It plays a vital role in the binding of *B. pertussis* and subsequent invasion of epithelial, ciliated and monocytic cells (103,198). FHA exhibits three specific attachment activities. The first is mediated by a cilia-specific carbohydrate binding site. A second heparin binding activity possibly mediates interactions of the bacteria with epithelial cells and extracellular matrices. The third activity involves an internal RGD sequence (Arg-Gly-Asp) which is thought to function in a carbohydrate recognition domain (CRD) and mediate bacterial/host interaction with the integrin receptors of mammalian cells (i.e. CR3 receptors in monocytes)

(76,103,155). It is thought that FHA may help initiate pertussis infection by recognizing and binding to sulfated glycolipids and proteoglycans commonly found on human cell surfaces. Although not considered a requirement for virulence, the high degree of bacterial/ host interaction mediated by FHA is likely critical in recognition of the bacteria by the host immune system and as such, has been included in many different acellular vaccine preparations (109,154,196,212,214). Purified extracellular *B. pertussis* FHA has been shown to increase the adherence of other bacteria to host cells, implicating this protein in the superinfections which can develop as a complication of pertussis infection (196,197).

The FHA structural gene, *fhaB*, resides in a 27 kb segment of contiguous DNA which also contains the *bvg* locus and the genes responsible for the production of *B. pertussis* fimbriae (218,219). The two fimbriae or pilin proteins (approximately 22 kDa, each) also play an important role in mediating adherence of the bacteria to respiratory epithelia, are present in all three species and are also immunogenic (119,120). As well, expression of fimbriae has been correlated with the later stages of a persistent infection in the infant mouse model (214). As such, the fimbriae are also considered to be important potential components of any *B. pertussis* vaccine (109).

The fimbrial genes *fimB-fimD* were once proposed to be involved in FHA production as well as fimbriae biosynthesis. Recently, however, *fimB-fimD* were proven to be involved in production of serotype 2 and 3 fimbriae (agglutinogens 2 and 3) only (218,219). A new gene , *fhaC,* identified downstream of *fimD,* was determined to be responsible for the FHA synthesis activity attributed to the *fim* genes. The interaction between *fhaC* and the N-terminus of FHA results in transport of FHA across the outer membrane. It is now suggested that *fimB-fimD* affect FHA production through polar affects on *fhaC* (218,219).

vi) Dermonecrotic Toxin (Heat Labile Toxin) - Dermonecrotic toxin is a 140 kDa polypeptide chain which is named for its ability to cause a characteristic skin lesion upon injection into test animals (102,224). There is a high degree of homology amongst all three *Bordetella* species in the gene structure, its toxin product and the appearance and production of the resulting lesion (133). In the past dermonecrotic toxin was often referred to as heat labile toxin in reference to its denaturation at 56°C. The role, if any, which it plays in *Bordetella* pathogenicity is still uncertain as the lack of this toxin in mutants does not affect virulence in the mouse lethality assay (214). Recently, a possible role as a vasoconstrictor has been suggested but proof to support this hypothesis is still in the initial stages (132,133,).

v) Pertactin - Pertactin is a 69kDa outer membrane protein (70 kDa in *B. parapertussis,* 68kDa in *B. bronchiseptica*) which contains an internal RGD sequence. As in FHA, this site is thought to interact with integrin receptors on eukaryotic cells and mediate adherence and internalization of the bacteria into its host cells (20,93,96,97). As previously mentioned this degree of interaction with the extracellular environment makes the pertactin highly immunogenic and an attractive prospect for inclusion in any vaccine (159,161). The protein is encoded by the *prn* gene (29,161).

vi) Brk Proteins - A recently discovered virulence factor, the bacterial resistance to killing (brk) proteins mediate the increased resistance of virulent *Bordetellae* to classical pathway complement-dependent killing by human serum (48). Fernandez and Weiss have characterized two proteins which are encoded by the *brk* locus. BrkA shows some sequence homology to pertactin and with two internal RGD sequences it is postulated to play a role in adherence and invasion. Initial data indicates that BrkB is a cytoplasmic membrane protein with possible transporter functions (48). The mechanism by which bacteria elude complement-mediated killing has not yet been fully elucidated.

vii) Flagella - While the molecules discussed to this point have been vir-activated gene (vag) products, the flagella genes are an example of a vir-repressed gene (vrg) (2,3). In an avirulent state the flagella genes of *B. bronchiseptica* are activated to produce flagella protein and the bacteria becomes motile (2,3). Motility would prove advantageous in an extraorganismal environmental niche such as a pond where the temperature is usually closer to ambient (20°C) than body (37°C) temperature. Of the three species, *B. bronchiseptica* is unique in its ability to grow under many different environmental conditions (60,223). Thus, coordinate regulation of the flagellar genes might prove useful in helping the bacteria to adapt to various surroundings.

As in the case of pertussis toxin, though only one *Bordetella* species produces the gene product, all three contain the genes (2,3). This interspecies homology is not limited to *bvg*-regulated molecules. It extends to biochemical and structural elements which are not regulated by the *bvg* locus.

Non-bvg regulated molecules

vii) Tracheal Cytotoxin - Tracheal cytotoxin (TCT) is a small (921 Da) soluble fragment of *B. pertussis* peptidoglycan which promotes ciliostasis of respiratory epithelium through postulated inhibition of DNA synthesis (59,164). TCT-mediated impairment of basal cell division and differentiation results in a long term inhibition of epithelial regeneration. Thus, the mucocilliary clearance mechanisms are arrested even after the bacteria have been killed with antibiotics. Through these actions tracheal cytotoxin is thought to inhibit neutrophil phagocytosis and hinder clearance of *B. pertussis*, mucus and other debris from the airway. It has been postulated that these actions play a major role in the induction of the paroxysmal cough which characterizes pertussis infection (38). It must be noted however, that avirulent organisms do not induce paroxysmal coughing in the coughing rat animal model regardless of the presence of TCT (141). It is therefore probable that some virulence-associated products are also involved in the production of the clinical symptoms.

viii) Porin Protein - The major porin protein is a prominent outer membrane component recently described in both virulent and avirulent *Bordetellae*. It is a heat-modifiable 40 kDa protein. All three species have been found to contain similar genes but the specific role of the porin channel in virulence has yet to be established (9,98).

ix) Endotoxin - The endotoxins of the *Bordetellae* share many similarities as well as numerous distinctions. The implications of these molecules to *Bordetellae* pathogenicity has not been established. My efforts towards clarification of this association forms the basis of the work presented in this thesis. The properties of the isolated endotoxins from *B. pertussis, B. parapertussis* and *B. bronchiseptica,* however, have been characterized in prior studies and will be examined in detail at a point further within this chapter.

Any examination of the importance of the many molecules associated with virulence is dependent on the possession of an animal model which adequately mimics the human condition. There are several animal models presently used in the study of *Bordetella* infection and pathogenicity. Each has distinct advantages as well as disadvantages.

3. Animal Models

B. pertussis is known to cause respiratory infections in mice, rats, rabbits, marmosets and great apes (147,173,207). The last three animal hosts have proven to be too large, expensive and high maintenance for most investigations. There are, however, several mouse and rat models commonly used in the study of *Bordetella* infection and disease.

The mouse intracerebral challenge model is the classic model used to assess the protectivity of each batch of whole cell vaccine. The mouse is first vaccinated intravenously then after one week a specified concentration of live organisms is injected into the base of the mouse's skull. Survival of the mouse indicates an effective vaccine (147,173). The largest drawback to this assay is the irrelevance of the challenge route. *Bordetella* colonization is almost always restricted to the upper respiratory tract (the exception being those rare cases of pneumonia and septicemia caused by *B. bronchiseptica*) (137,203).

The aerosol/intranasal mouse challenge model better simulates the actual disease path. Infant mice are exposed to *B. pertussis* organisms through sonic-induced aerosols or manual application of droplets (173,180,214). Aerosol delivery tends to be more uniform. A problem with the model is the difference in clinical presentation of pertussis in mice as opposed to humans. Infection of mice produces neither the characteristic leukocytosis nor cough. As well, the end result of infection is often death in this model. Indeed, LD50 (the bacterial dose capable of causing death in 50% of mice tested) is often the parameter assayed in comparative studies (48,209,214).

Symptomatically, the coughing rat model better approximates human disease (68,141,180). It involves the intrabronchial placement of *Bordetella* encased in agarose beads. A marked leukocytosis is notable and the characteristic cough is often present in infected rats. A possible problem is the site of infection. As mentioned, colonization of humans is almost always limited to the upper respiratory tract (137,203).

4. Bacterial Growth and Disease

Although they differ substantially in colonial morphology and growth characteristics, all three species of *Bordetella* are capable of acting as human pathogens (147,207,223). *B. parapertussis* requires two days to produce small colonies which are notable in the browning of surrounding media caused by a unique tyrosinase activity. It is known to commonly cause a less severe pertussis-like disease in both humans and sheep (due possibly to its lack of pertussis toxin) (99,101,147,223). Less than 20% of clinically recognized cases present with typical pertussis-like symptoms. The mildness or complete absence of symptoms causes problems in epidemiological studies of *B. parapertussis* infection. *B. parapertussis* is usually diagnosed only in cases of co-infection with *B. pertussis* or in abnormally severe infections (99,100). This presents researchers with a deceptively low concept of its prevalence within the population. Antibody studies have indicated that up to 50% of the population has had some form of *B. parapertussis* infection.

B. bronchiseptica is the least fastidious of the three *Bordetella* species. It is motile, nutritionally undemanding and produces colonies (0.5 - 1.0 mm) within 24 hours of subculture. *B. bronchiseptica* is the causative agent of a number of upper respiratory ailments in various hosts; kennel cough in dogs, as well as diseases in guinea pigs and in very rare cases, humans. It is an important cofactor in the development of atrophic rhinitis in pigs (60,223). As well, *B. bronchiseptica* has recently emerged as a cause of bronchopneumonia in severely immunocompromised humans, notably AIDS patients (223).

It is the disease caused by *B. pertussis*, however, that has been best documented as a more common cause of severe human illness. *B. pertussis* was first isolated by Bordet and Gengou at the turn of the century (18). It is the primary cause of clinical pertussis or "whooping cough" in man and doesn't have any other known reservoir or environmental niche (137,147,207). It is nutritionally fastidious and produces small colonies (1 mm) within 3 days of culture.

After an asymptomatic incubation period of 1 to 2 weeks the disease progresses through three stages (137,147,203,207). The initial cattarhal ("prodromal") stage resembles a common cold, presenting symptomatically with low-grade fever, a dry non-productive cough and general malaise. Ninety percent of culture positive nasopharyngeal swabs are collected from patients in this stage of infection. The second "paroxysmal" stage occurs approximately one week later and defines the disease. The patient often (but not necessarily always) suffers severe paroxysms of mucous-heavy coughing which end in vomiting and/or a characteristic "whoop" upon inspiration. Fever is very rare at this point in uncomplicated pertussis. This stage generally lasts no longer than two weeks and the percent of culture positive samples decreases dramatically, indicating clearance of the bacteria, itself. A notable clinical presentation during this stage is marked leukocytosis.

Within four weeks the disease has usually progressed to the convalescence stage which can last for up to 6 months. A secondary immunological challenge at this point can cause a reoccurrence of paroxysmal coughing. Most patients recover fully from their illness. Complications may arise, however, due to injuries incurred as a result of the paroxysmal coughing or onset of a secondary infection in the immunocompromised host. The violent, prolonged coughing spasms can result in apnea induced convulsions or cyanosis as well as anoxia, cerebral or subconjunctival hemorrhage, umbilical or inguinal hernia and rectal prolapse. The onset of fever often indicates a secondary infection. This can result in otitis media, aspiration bronchopneumonia, or reoccurrence of a quiescent disease such as measles or tuberculosis (101,137,147,206,207).

In the undervaccinated, malnourished third world the high mortality rate of pertussis is due, in part, to the young age of its most common victims. While vaccinated individuals and adults usually experience only minor flu-like symptoms, unvaccinated children under 8 years of age more often experience the full case definition of the disease (19,100,101). The presence of undiagnosed disease poses a constant threat to the youngest and weakest members of a population. Misdiagnosis and underestimation of disease seriously erodes the "herd" immunity which protects the unvaccinated by allowing a bacterial reservoir to exist undetected within a population. Overestimation of disease leads to imprudent use of antibiotics and likewise threatens disease control through the introduction of antibiotic resistance. The current methods of clinical diagnosis contain a substantial risk of misdiagnosis (47,50,99).

Briefly, a nasopharyngeal swab is smeared to a glass slide and tested for the presence of *B. pertussis* using a polyclonal antibody direct fluorescent antibody (DFA) assay. A separate swab is cultured to a Regan-Lowe plate for possible retrieval of the organism. A problem with this protocol is the large number of positive DFA results in comparison with the small number of positive culture results (47). Either the DFA is yielding false positive data or the culture results often appear to be falsely negative. Investigations in our laboratory found that both incidences were occurring. A new *B. pertussis* monoclonal DFA greatly reduced the incidence of cross-reactivity. It is hoped that the addition of a *B. parapertussis* monoclonal antibody will also aid in epidemiological classification of each case. Presently, adults with undiagnosed disease are a major source of transmission to the younger, more vulnerable members of a population (49).

An improved diagnostic system would aid in controlling the spread of disease caused by the *Bordetellae.* As well, dependable epidemiological information is required in order to ensure the continued efficacy of the whole cell vaccine serotype in use in a given area (31,65).

5. Vaccines

As *B. parapertussis* is considered to cause a mild disease there are presently vaccines available for *B. pertussis* and *B. bronchiseptica,* only. Considering the high degree of homology amongst the surface components of the *Bordetellae,* however, it is not unreasonable to conjecture that the whole cell vaccines currently used to protect against *B. pertussis* and *B. bronchiseptica* may provide some cross-protection against *B. parapertussis* (78,79). The concentration of pertussis toxin and endotoxin are monitored in each vaccine batch by several methods; notably the in vitro CHO cell assay and the limulus amoebocyte lysate gelation test, respectively. As previously mentioned the mouse intracerebral challenge model is used to assay vaccine efficacy. The mouse weight gain assay is utilized to assess overall vaccine toxicity (28,85,126).

The whole cell vaccine presently in use has been the source of controversy for over two decades. The DPT (diphtheria-pertussis-tetanus) vaccine consists of killed *B. pertussis* organisms in combination with diphtheria and tetanus toxoids. Much of the concern stems from reports of side-effects ranging from fever, and soreness at injection site to permanent neurological sequelae (31,32,64,165,166). Although there is still extensive debate as to the validity of the latter effects, the incidences of the former, less serious, side-effects are well documented and occur with regularity. Indeed, the whole vaccine has been implicated in a high percentage of fevers in children under one year of age (58).

Aside from the regrettable distress caused to child and parent the possibility of misinterpretation of these minor side-effects as an indicator of the subsequent occurrence of less transient sequelae can lead to termination of the vaccination schedule. Compliance is a major problem in administering the four injection schedule of DPT vaccination (49,58,65,147,165,222).

In the face of the controversy presently surrounding the whole cell vaccine three main options are left to health authorities; 1) discontinue vaccination altogether, 2) produce and administer an acellular vaccine or 3) modify the current vaccine to a less reactive state. Discontinuation of the DPT vaccination is not a viable alternative. In the past 2 decades low compliance due to media-driven concern over vaccine safety has proven that pertussis is still very much a threat to public health. Outbreaks have occurred wherever vaccination rates have dropped (49,65,137,222). Whereas the incidence of neurological damage after receipt of the vaccine has been estimated as 1 in approximately 200,000 doses, the possibility of neurological sequelae due to the disease is approximately 1 in 2,000 cases. As well, again, it has been established in several studies that the importance of the "herd" immunity which has built up over several decades is protective, particularly to infants who have not yet been vaccinated (49,65,222). A vaccine is definitely preferable to the alternative of the disease and its associated morbidity.

It would be highly desirable to produce an equally effective but less reactive vaccine. Many acellular "component" vaccines are presently in development, however, the complexity of both the disease and the immune response to the numerous toxins and factors of the *Bordetellae* has rendered the development of an acellular vaccine both expensive and complicated (109,162,172). The added expense and possible compromise in efficacy which these new vaccines represent could be circumvented by a less reactive version of the whole cell vaccine.

Given the documented in vivo toxicity of endotoxin, in general, and *B. pertussis* LOS in particular, it is the presence of *B. pertussis* lipooligosaccharide in the whole cell vaccine to which many of the minor complications including fever, restlessness, local soreness and swelling at the injection site, have been attributed (23,28,64,85). This correlation has been strengthened by the lack of such symptoms in acellular vaccines containing pertussis toxin and other molecules (172).

Recent data with synthetic analogues and acyloxyacyl hydrolase (AOAH) LPS derivatives suggest that much of the discomfort associated with the whole cell vaccine could possibly be alleviated with the modification of the *B. pertussis* endotoxin or lipooligosaccharide (LOS), specifically the lipid A moiety (43,44,182,194). However, most investigations of lipid A biosynthesis and structure/function relationship have centered on enteric species, and virtually nothing is known of the organization or regulation of either the structural or regulatory genes responsible for *B. pertussis* LOS biosynthesis. The studies detailed in this thesis represent the initial examinations of the genetic organization, function and clinical relevance of LOS in the *B. pertussis* outer membrane. As the host recognizes endotoxin as a component of the outer membrane rather than an isolated molecule, studies of clinical relevance require some knowledge of the molecules and mechanisms which are interacting with cell-presented endotoxin.

B. THE OUTER MEMBRANE

The Gram negative cell envelope has been resolved into three distinct "strata" which proceed from the extracellular to intracellular environments: an outer membrane anchored to a peptidoglycan (PG) layer and an underlying cytoplasmic membrane (170,203).

The outer membrane consists of an inner and outer leaflet. The inner leaflet is similar in composition to the cytoplasmic membrane while the outer leaflet possesses a number of distinct characteristics which define its unique properties and to a large part, the unique properties by which Gram-negative organisms have been differentiated from Gram-positive organisms. Each of the many components which constitute the outer membrane contribute to these distinct characteristics (71,106,134,170,203).

1. Structure

i) Lipoprotein - The presence of lipoprotein is one characteristic which distinguishes Gram-negative organisms from most Gram-positive bacteria. Two different types of lipoprotein have been found in the Gram-negative outer membrane. Braun's lipoprotein is a small (7.2 kDa) protein initially discovered in high concentration (7.0×10^5 molecules/cell) in *E. coli* (21,22). Two thirds of molecules are "free" in the outer membrane while the remaining one third are covalently linked to the peptidoglycan layer in two ways; an amide bond from the lipoprotein lysine to the ε-amino group of the diaminopimelic acid of peptidoglycan and a lipoprotein diglyceride linked as a thioester to cysteine (21,22,74).

The second group of lipoproteins is termed the peptidoglycan-associated lipoproteins (PAL) (117). These lipoproteins are larger (21 kDa for OprL of *P. aeruginosa* and Protein 21K from *E. coli*), vary in membrane concentration (OprL is in high abundance, Protein 21K is found in low concentrations) and are associated with the peptidoglycan by noncovalent linkages exclusively. Lipoprotein is important to the structural integrity of the cell as well as the barrier functions of the outer membrane.

ii) Peptidoglycan (murein) - Peptidoglycan forms an intermediate layer between the cytoplasmic and outer membranes. It is linked to the outer membrane both covalently, by lipoprotein as well as other proteins such as the major outer membrane protein (MOMP) of *Legionella,* and noncovalently, by various porin and OmpA-like proteins (86). Basically, peptidoglycan is a heteropolymer which is constructed from glycan strands of N-acetylglucosamyl-N-acetylmuramic acid that are crosslinked by short peptides. UDP-N-acetylglucosamine is a common precursor used in both peptidoglycan and endotoxin biosynthesis (140). Over a hundred variations in binding and substitution characteristics have been documented in studies of peptidoglycan structures. In Gram-negative bacteria the function of peptidoglycan is more in maintenance of cellular structure and rigidity rather than as a principle barrier to the external environment as is the case of the much thicker peptidoglycan multilayer of Gram-positive organisms (170).

iii) Phospholipids - Most of the outer membrane phospholipid content is found in the inner leaflet as endotoxin is found in place of much of the outer leaflet phospholipid. The major phospholipids of bacterial outer membranes are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (94,203).

iv) Proteins - Unlike the cytoplasmic membrane, the Gram-negative outer membrane contains a small number of "major" proteins which are readily identifiable from the overall protein pattern. Many of these major outer membrane proteins (OMPs) play important roles in the molecular transport and uptake of metabolites (203).

"Porin" proteins are capable of forming pores through the outer membrane which have defined properties and molecular exclusion limits for a number of hydrophilic and hydrophobic compounds (131,134,203). They are the most abundant proteins in *E. coli*, in terms of mass and have been postulated to interact noncovalently with the underlying peptidoglycan (163). There are 2 general classes of porin proteins. Nonspecific porins form nonspecific hydrophilic pathways, have limited ion selectivity and a size exclusion limit of between 600 - 1400 Da. Specific porins have decreased selectivity for small molecular weight molecules but increase facilitated diffusion of specific larger molecules, ie. LamB porin is selective for maltose, Tsx porins are specific for nucleosides (69,71,193).

OmpA is a protein of approximate molecular weight of 35 kDa which is often associated with lipoprotein (106,202). It is a receptor for certain phage and has been postulated to function in stabilizing the membrane and as a component in the conjugation process. The notable importance of this protein to the cellular dynamics will be discussed in the next section.

v) Cations - Cations are the "glue" which bind together the Gram-negative outer membrane. Studies have discovered a number of inorganic ions (Monovalent (Na^+, K^+) and divalent ($Mg2^+$, $Ca2^+$) cations as well as organic cations (putrescine, spermine, spermidine, and ethanolamine) closely associated with the outer membrane (35,36,55). Divalent cations, in particular have an important function in outer membrane integrity and stability due to the strong affinity of endotoxin for such ions (176,201,202).

vi) Endotoxin - Endotoxin is of primary importance to the organization and function of the outer membrane. This relationship is central to the theme of this thesis. The significant contribution of endotoxin to the function and structure of the outer membrane is described below. The structure and function of the isolated endotoxin molecule will be dealt with in detail in the following section.

Overall, the Gram-negative cell envelope is technically defined as: "a complex structure consisting of an asymmetric outer membrane possessing lipid A-anchored lipopolysaccharides in an outer leaflet of a bilayer membrane containing trans-membrane porin protein channels, stabilized by the covalent linkage of a lipoprotein with thioester-bonded diglyceride at the N-terminal cysteine residues and a C-terminal lysine amide linked to the ε-amino group of α,ε-diaminopimelic acid in the peptidoglycan"(170).

2. Dynamics (The Principle Interactions of Outer Membrane Components)

i) Endotoxin/Phospholipid - In the outer membrane the biosynthesis of phospholipids is in a sensitive dynamic balance with that of endotoxin through the use of a common precursor. *R*-3-hydroxymyristoyl-ACP is situated at a biosynthetic branchpoint; it can be attached to the lipid A of endotoxin or elongated to palmitoyl-ACP and used in phospholipid biosynthesis (37). A decrease in endotoxin production due to treatment with external agents such as detergents or the introduction of a mutation, has been demonstrated to result in a compensating increase in phospholipid production through a "switch" in the biosynthetic destination of *R*-3-hydroxymyristoyl-ACP from endotoxin to phospholipid production.

The physical properties of phospholipids, however, differ significantly from those of endotoxin. The endotoxin molecule is larger and more hydrophobic than phospholipids since phospholipids have 2 attached fatty acids, while endotoxin commonly has 6-7 covalently linked acyl chains, including characteristic secondary acyloxyacyl chains (71,134,170,203). The fatty acids of endotoxin are all saturated rendering the hydrocarbon interior of the outer leaflet almost crystalline in its rigidity and the tight interaction of adjacent endotoxin molecules is stabilized by divalent cation bridges between the negatively charged groups of endotoxin.

Clearly the two molecules can not be interchanged without the occurrence of dramatic alterations in outer membrane structure and function. The strength of the phospholipid/endotoxin relationship is far weaker than that of endotoxin with endotoxin. The cell becomes hypersensitive to detergents, hydrophobic antibiotics and cation chelators, such as EDTA (94,195).

The presence and quantity of phospholipid in the outer membrane is dynamic. A "normal" intact outer membrane includes very little phospholipid. If, however, normal endotoxin/endotoxin or endotoxin/protein interactions are disturbed (ie. by mutation as is the case in deep rough mutants of Salmonella and *E. coli*, or by displacement/replacement of ions with chelators or detergents) and either endotoxin or protein is lost the phospholipid concentration increases in a compensatory effort to retain the structural integrity of the outer membrane (71,134,170,203). In addition to the endotoxin/phospholipid interactions this creates patches of "pure" phospholipid bilayer in the outer membrane which are far more vulnerable to hydrophobic agents than the remaining regions composed of endotoxin. Thus, the barrier properties of the outer membrane falter and the cell becomes more sensitive to a host of hydrophobic agents (5,84,160,171,184).

ii) Endotoxin/Protein - As is the case with phospholipids, the role and presence of proteins in the outer membrane is variable and dynamic. The decrease in saccharide chain length in deep rough mutants of *Salmonella* *typhimurium* and *E. coli* results in a loss of associated protein which in turn results in an increase in phospolipid concentration and concomitant increase in sensitivity to hydrophobic agents (160,171). Thus, although there is no notable loss of endotoxin in these strains, the alteration alone is significant enough to destabilize the endotoxin/protein interactions. The assembly and organization of the outer membrane is thought to be dependent on specific endotoxin/protein interactions. Deep rough endotoxin mutants do not interact properly with proteins due to deficiencies in the carbohydrate chains and/or phosphate groups of the endotoxin molecule. The disruption in outer membrane organization occurs at a specific point of truncation. In *S. typhimurium*, Rc mutants which are missing approximately 80% of the carbohydrate moiety of their endotoxin are normal in their susceptibility to hydrophobic agents such as novobiocin (203). An Rd mutant strain of *Salmonella typhimurium* has a single glucose residue less than Rc yet is notably sensitive to hydrophobic agents (161,171). The sensitive RcP- mutant strain of *S. minnesota* differs from the native structure in the loss of a single phosphate. The point at which structural alterations of endotoxin result in disruption of the outer membrane differ between species but appear to be extremely specific.

Many proteins have demonstrated structural and functional associations with endotoxin. The *E. coli* OmpA protein is structurally and functionally associated with endotoxin. In experiments by Schweizer et al., both isolated and cell membrane-presented OmpA was protected from proteolytic cleavage by association with endotoxin (178). In addition, OmpA requires endotoxin for F-plasmid mediated conjugation as well as receptor functions for the Tull and K3 phages (39,110,183,205). The phage receptor activities of the OmpF and OmpC porins also require endotoxin with a complete core moiety (39,203,205). The H1 protein in *Pseudomonas aeruginosa*, produced in response to a decrease in Mg^{2+}, substitutes for Mg^{2+}, binds and protects the cationic sites of endotoxin in an effort to stabilize the outer membrane (69,70,71,203). There is also evidence that the Opa proteins of *Neisseria gonorrhoeae* form tight intercellular junctions by binding to the carbohydrate moiety of endotoxin molecules of adjacent cells (16).

iii) Endotoxin/Endotoxin (Endotoxin/Cations) - Divalent cations such as Mg^{2+} and Ca^{2+} decrease (but do not entirely neutralize) the electronegative repulsion in adjacent endotoxin molecules by binding to phosphate and carboxyl groups (ie. on KDO sugar of the endotoxin core) (69,176). As such these cations are essential to the integrity of the membrane. Chelation of these ions with EDTA or substitution with polycationic agents such as polymyxin B results in increased cellular permeability, leakage of periplasmic enzymes, structural alterations and loss of endotoxin from the membrane(203). Alternatively, addition of divalent cations such as Mg^{2+} can tighten the molecular interactions and reverse the alterations in membrane structure and function (30,128,156,186).

The outer membrane exists as a dynamic electronegative system where cations are in rapid, constant diffusion across the membrane surface. A large polycation, such as polymyxin B, causes the localized exclusion of native cations and results in the stable neutralization of a localized area. This in turn encourages the interaction of other polycations with regions adjacent to these newly stabilized regions of the outer membrane. The disruption in charge fluidity and introduction of more structurally rigid molecular interactions leaves electroneutral gaps and cracks between molecules which commonly associate by such ionic "bridges" (endotoxin/endotoxin, endotoxin/anionic proteins, etc) (170,201,202,203).

Thus, the properties of the bacterial outer membrane are dictated, to a large degree, by the interactions of its molecular components. In turn, the pathogenic potential of a bacteria is dictated by the interactions of the outer membrane with its extracellular environment, the host immune system. The following section demonstrates the effect that alteration of outer membrane components such as endotoxin has on outer membrane structure and bacteria/host interactions.

3. Interaction Of The Outer Membrane With The Extracellular Environment

i) Antibiotics

Hydrophobic - The disorganized membrane of deep rough endotoxin mutants are also susceptible to passage of hydrophobic antibiotics through membrane cracks or phospholipid bilayer patches. These include macrolides, novobiocin, rifamycin, actinomycin D and hydrophoboic penicillins such as nafacillin (160,171).

Hydrophilic - In deep rough mutants a decrease in sensitivity to hydrophilic antibiotics such as penicillin, ampicillin, cephalothin and teracycline has been observed. This phenomenom has been postulated to be due to a loss of the porin proteins which would normally allow access of these agents to the cell interior. The transport of bulky, slightly hydrophobic antibiotics are also greatly affected by alterations in the number or nature of porin channels in the outer membrane (160,171).

iii) Components of Human Sera

Human sera contains a number of components which interact with the Gram-negative outer membrane, notably the endotoxin molecule (128,156). The host produces specific antibodies to the exposed, immunogenic carbohydrate moiety of the endotoxin which subsequently target the cell for lysis by professional phagocytes or complement. Endotoxin is capable of activating complement in three documented ways: 1) through the classical pathway in the presence of antibody, 2) through the classical pathway in the absence of antibody (by interacting directly with the C1q component), as well as 3) through the antibody-independent alternate pathway (55,56,122). Interestingly, the same antigenic O-chains which originally target the cell for destruction ultimately serve to protect the cell from lysis by serum components. The hydrophilic sugars provide a physical barrier to attachment of the complement-mediated attack complex to the cell membrane.

iii) Detergents, Dyes and Other Molecules

As previously mentioned, the outer membrane when disrupted, as in deep rough endotoxin mutants, is sensitive to hydrophobic detergents such as SDS, bile salts and Triton X-100 as well as cationic detergents such as polymyxin B (70,203). The disrupted membrane is similarily permeable to hydrophobic dyes such as eosine, brilliant green, methylene blue and gentian violet. Other molecules to which these mutants show increased sensitivity include fatty acids, phenol and polycyclic hydrocarbons (186,203).

iv) Other Stimuli Which Alter Outer Membrane Structure and Function.

The presence or absence of many outer membrane proteins is dependent on external stimuli. *E. coli* grown in maltose-rich media greatly upregulates the concentration of the LamB porin (106,193). Under iron -starvation conditions, *E. coli* increases the outer membrane concentration of iron - uptake proteins while phosphate starvation results in a decrease in the PhoE porin (138). The upregulation of phospholipases (ie. phospholipase A1) causes increased hydrolysis of phospholipids and subsequent reorganization of the membrane (106,204).

A more drastic example of the reactive properties of the outer membrane are the outer membrane receptors of coordinate regulation systems in pathogenic bacteria such as the *Yersinia* and the *Bordetellae* (191). Antigenic modulation is induced through outer membrane perception of environmental signals such as an change in temperature or nicotinic acid concentration. In response, the host of proteins which characterize the cellular shift from virulence to avirulence (and back) are upregulated while others are simultaneously downregulated. The effect of endotoxin on such as system as is found in the *Bordetellae* remains to be clarified.

The outer membrane undergoes gross structural disruption as the result of alterations in the structure of the endotoxin molecule. Drastic changes in the functional properties of the outer membrane occur as the result of the reorganization of macromolecules, the occurrence of phospholipid bilayer patches, and/or the development of "cracks" in the normally tight associations between endotoxin molecules (and/or endotoxin and acidic proteins). The structure and physical properties of the endotoxin molecule govern the extent and limitation of these alterations for each bacterial species.

C. ENDOTOXIN

1. Historical Perspective and General Structure

In 1856 Dane P. L. Panum first attempted to characterize "putrid poison" the toxic, pyrogenic agent found in putrid or septic fluids (139). It was not until Robert Koch devised protocols to generate pure bacterial cultures however, that the isolation of this substance became possible. It was Richard Pfeiffer, a student of Koch's, who first identified an "endotoxin" of the cholera bacillus which, in contrast to previously characterized "exotoxins" remained attached to and an integral part of the bacteria (145). Within the same period, Eugenio Centanni was describing "pyrotoxina bacteria", a group of organisms characterized by their production of a carbohydrate-rich, protein-poor, pyrogenic, cell-bound toxin (27). This endotoxin has since been found to be an integral component of all Gram-negative microbes and the principle cause of gram-negative sepsis (217).

The chemical characterization of endotoxin, however, remained stalled until Andre Boivin developed a trichloroacetic acid (TCA) method of extracting such sugar-fatty acid complexes from the bacterial cell (17). Walter Morgan further perfected the extraction procedure with the use of a milder diethylene glycol protocol (121). It was Otto Westphal, however, who, in 1952, developed the hot phenol/water extraction method for endotoxin which is still in use today (215,216). With significant amounts of highly purified endotoxin available, research on the biological properties and chemical structure could proceed. The pyrogenic, toxic and immunological activities of endotoxin were established over the next several decades through the work of numerous researchers (104,113,158). The discovery of "rough" chemotype variants led to initial elucidation of the coarse structure-function relationship of endotoxin. These mutants were found to be lacking a large amount of the carbohydrate of the "smooth" parental strain. C. Galanos first developed an extraction protocol utilizing phenol, chloroform, and petroleum ether (PCP) to obtain better yields of the rough chemotypes (53). He then created a series of mutants of *Salmonella minnesota* which produced LPS molecules with progressively truncated endotoxin. They were termed Ra, Rb, Rc, Rd, and Re, respectively from least to most truncated. The deepest mutation, found in the Re variant, produced an endotoxin consisting of lipid A and three ketodeoxyoctonic acid (KDO) units. This has since been found to be the minimum endotoxin structure possible for production of viable bacteria. Lipopolysaccharide (LPS) and its associated proteins compose the greater part of the outer leaflet of the outer membrane and further truncation produces an LPS molecule which is physically incapable of maintaining a stable lipid bilayer (203). Detailed analysis of the R-mutants established the presence of three distinct sections of endotoxin; the exposed, immunodominant set of repeating sugars termed the O-side chain; a core region containing several characteristic rare sugars (substituted heptoses and KDO); and a fatty acid-rich lipid A region in which rested the toxic and pyrogenic properties of endotoxin. The name lipopolysaccharide (LPS) was firmly established to describe this toxin. The toxicity of the lipid A region was further established and examined through studies involving synthetic analogs of LPS and lipid A as well as chemical fractionation of native molecules (157,181). The presence of secondary acyl chains as well as attached phosphate groups were determined to be essential for lipid A toxicity in enteric bacteria.

The study of LPS in nonenteric species has closely followed the elucidation of the structure and function of the LPS of enteric bacteria. There are slight but notable differences between the endotoxin of the two groups In the lipid A structure the arrangement of secondary acyl chains around the di-glucosamine backbone is generally symmetrical in nonenteric endotoxin while the secondary fatty acids are distributed asymmetrically in enteric LPS (26,28).

2. Biosynthesis and Genetic Organization

Studies in Salmonella and *E. coli* indicate that endotoxin is synthesized on the internal side of the cytoplasmic membrane as 2 components; core/lipid A and O-chain (108,153). The two components are then transported separately to the external or periplasmic leaflet of the cytoplasmic membrane, ligated together and "accessorized" with additional ethanolamine, sugar groups etc. Most of the studies completed on endotoxin biosynthesis have been done in enteric genera such as Salmonella, Shigella, and *E. coli*. "Rough" mutant strains contain incomplete LPS (typically truncations in the O-side chain and core moieties).

Genetic characterization of a series of rough mutants has resulted in the documentation of a number of distinct gene "clusters" or "blocks", each specific for the construction of a particular endotoxin component (108,150,153). The *rfa* cluster at approximately 80 min, contains all transferase genes required for core synthesis. As well, *kdtA,* a gene adjacent to *rfa* produces the KDO transferase which adds KDO molecules onto the incomplete lipid A molecule. The *E. coli rfa* cluster contains three sequential operons. The first encompasses *kdtA* plus an open reading frame entitled *kdtB*. The second operon encodes genes required for heptose synthesis and the third operon consists of genes required for outer core synthesis. The genes responsible for lipid A biosynthesis, *lpxA, lpxB, lpxD* and *envA* (*lpxC*) map to a genomic region at approximately 2-4 min. The *rfb* gene cluster which produces transferases responsible for O-unit synthesis often contains the *rfc* gene which encodes the O-polysaccharide polymerase. All species investigated were enteric or have native lipopolysaccharides which contain O-unit polysaccharides. No nonenteric species with a native lipooligosaccharide phenotype, such as is found in *B. pertussis,* has been examined in the same manner.

3. *Bordetellae* Endotoxin

i) Structure

The lipooligosaccharide (LOS) of *Bordetella pertussis* largely conforms to the classic enteric lipopolysaccharide structure but the O-side chain portion is truncated to 3 sugars (hence the absence of a "ladder" motif on silver stained SDS PAGE) (Figure I.1). As well, *B. pertussis* LOS is structurally distinct in its reduced heterogeneity (ie. cellularly conserved structure), significantly increased degree of phosphorylation and its possession of an unusually large number of amino sugars with non-acylated NH_2 groups (88). The lipid A of *B. pertussis* is also distinct in its possession of a very short chain (C_{10}) fatty acid not commonly found in enteric species (26,28).

The LOS of *B. pertussis* is heterogeneous within an organism presenting as two distinctive low molecular weight bands on SDS-PAGE and eluting in two separate peaks off a hydroxylapatite column (90,142). The hydroxylapatite peaks, LPS-I and LPS-II, were initially thought to correspond with the two bands observed on SDS-PAGE but recent unpublished observations by LeDur and Chaby have denied this correlation. The difference between LPS-I and LPS-II has been determined to be the addition of a phosphate group to the KDO moiety in LPS-II (28). The SDS-PAGE bands were designated LOSA and LOSB respective of their decreasing molecular weight. Recent studies by Caroff et al. have produced evidence that the faster migrating LOSB band is the result of the truncation of three sugars from the nonreducing end of the larger molecular weight LOSA species. It is these three sugars that Caroff's group has designated the "O-chain" of B. pertussis (25). The heterogeneity as observed by SDS-PAGE analysis is largely conserved from virulent to avirulent organisms (a slight increase in LOSB concentration in avirulent strains has been reported by Martin et al.) and while considered the wildtype phenotype, there do exist mutant phenotypes where LOSB, alone, is present (111). *B. pertussis* LOS consistently exhibits both in vivo and in vitro properties (Table I.1) (208).

The endotoxins of the three *Bordetella* species under study share many common features. The lipopolysaccharide of *B. bronchiseptica* shares common structural features with the endotoxins of each of the other two species (25,89). Its core carbohydrates are very similar to those of the lipooligosaccharide of *B. pertussis* while the lipopolysaccharide of *B. parapertussis* shares the same O-side chain structure. The structure of the endotoxins of *B. bronchiseptica* and *B. parapertussis* differ most distinctly from that of *B. pertussis* in that the former species contain full O-side chains of approximately 20 monosaccharide residues (25,89).

As mentioned, the lipopolysaccharide of *B. bronchiseptica* has a core structure which possesses a very high degree of homology with that of *B. pertussis.* Unique to the *B. bronchiseptica,* however, is its ability to vary its LPS core structure with virulence (144). Two higher molecular weight bands, deemed LPSA and LPSC are present in the virulent phenotype while two bands of slightly lower molecular weight, LPSB and LPSD, can be seen in the avirulent organism. Present knowledge indicates that the O-side chain remains unchanged.

ii) Biological Activity

The biochemical, immunological and biological properties of the isolated endotoxins of the *Bordetellae* have been well characterized in several studies. Unfortunately, endotoxin concentrations, assay conditions, as well as *Bordetella* and enteric reference strains have varied widely from study to study. This makes the total body of assay data difficult to compare, however a synopsis of comparable assays is presented in Table I.2.

The pyrogenicity, toxicity and immunogenicity of *Bordetella* endotoxin was initially established in a 1960 study by MacLennan et al (107). The endotoxin concentrations utilized in these investigations were considerably higher than those used in later studies, due to the stated desire to establish, definitively, the properties of *Bordetella* endotoxin in comparison with those of LPS isolated from enteric species. Toxicity of endotoxins from *B. pertussis, B. parapertussis,* and *B. bronchiseptica* were investigated through quantitation of lethality due to intravenous injection of the endotoxin into white rabbits and intraperitoneal injection into mice, white rats, and guinea pigs. All endotoxins displayed toxicity, however, *B. bronchiseptica* endotoxin was found to have the highest toxicity with *B. parapertussis* LPS being slightly less toxic and *B. pertussis* LOS less so than the others. As well, the endotoxins of all three species exhibited pyrogenicity in rabbits equal to that of endotoxins from species of the *Enterobacteriaceae* and *Pasturelleae*. Several subsequent studies by disparate investigative groups have further elucidated the biological properties of *Bordetella* endotoxin (1,4,6,7,11,28,88,125,126,151,152,182,208). It is clear that the isolated endotoxins of the *Bordetellae* are comparable to their enteric counterparts in most immunological and toxicicological assays. The few exceptions to this rule are detailed further in this section.

Initial fractionation studies of *B. pertussis* LOS reported the existence of a major nontoxic lipid A component as well as a minor lipid X moiety which possessed all the examined properties of enteric endotoxin (11,28). It was eventually determined that the reported "lipid A" was in fact a degraded form of the true lipid A molecule which had been stripped of several phosphate and acyl groups. A number of studies have since proven the absolute requirement of these groups to endotoxin toxicity. The toxic "lipid X" was, in fact, all that remained of the undenatured lipid A. The use of a milder protocol to separate lipid A from the core sugars involving SDS greatly decreased this degradation. Studies by the same group have since proven the properties of the lipid A moiety of *B. pertussis* to equal the toxicity of enteric lipid A groups (28). (The use of the term "lipid X" was an unfortunate coincidence and has no correlation with the lipid X biosynthetic precursor molecule subsequently identified by Christian Raetz's group.)

The final toxicity attributed to *B. pertussis* lipid A better corresponds with the previous studies of the complete endotoxins of all three *Bordetellae* species which determined them to possess most of the properties of enteric lipopolysaccharides (Table I.1) (208). Studies found all *Bordetellae* endotoxins to be capable of inducing pyrogenicity in rabbits, lethal toxicity in galactosamine-sensitized mice, mitogenicity in C3H/HeN spleen cells, macrophage activation and tumor necrosis factor secretion. While *B. pertussis* and *B. bronchiseptica* were as effective as the *E. coli* standard, the LPS of *B. parapertussis* was found to be slightly less effective at producing these effects. As well, investigation of the properties of *B. pertussis* determined its unusual ability to induce nonspecific resistance to viral and bacterial infection (11,28).

Other discrepancies have been noted in comparing the properties of *Bordetella* endotoxin to those of enteric species. Several strains of *B. pertussis* have been found to have decreased activity in the endothelial cell adherence assay and increased antitumor activity. Both MM46 mammory carcinoma and MethA fibrosarcoma were found to be highly susceptible to *B. pertussis* LOS at concentrations much lower than those required of *E. coli* endotoxin and with far less associated toxicity. Based on this data, Watanabe et al have proposed a potential clinical role in cancer therapy for *B. pertussis* LOS (208). As well, *B.*

pertussis LOS has been found to act as a mitogen on endotoxin-nonresponder C3H/HeJ spleen cells (88). The spleen cells of C3H/HeJ mice are nonresponsive to most endotoxins due to the expression of a mutant allele (Lps^d) at the chromosome 4 locus. The exact product and mechanism of tolerance which is mediated by this locus remains unknown (88). The ability of *B. pertussis* LOS to elicit a response has been attributed to the four terminal sugars in the core region which are immediately proximal to the lipid A moiety.

Aside from the exceptions noted, numerous studies have proven *Bordetella* endotoxins to be comparable to classic enteric LPS in toxicity and immunological characteristics. Although the LOS of *B. pertussis* can not, in itself, confer protection in the infant mouse model it does elicit high IgM, IgA, and IgG responses in both vaccinated and infected individuals (10,28,130). It is very possible that *Bordetella* endotoxin may play an important role in protection through presently undefined associations with other surface components (28,136).

D. RATIONALE FOR EXPERIMENTATION

The diseases caused by the *Bordetellae* as well as the subsequent host immune response are extremely complex, mediated as they are by such a large number of toxins and virulence factors. Many excellent thorough studies have examined the functions and properties of isolated molecules, as outlined above. No individual factor or toxin, however, has been found which adequately accounts for the clinical symptoms engendered by bacterial infection of a susceptible host.

The *Bordetellae* are distinct from many other bacteria in that the disease produced by the whole cell is more a product of the interactions and associations of individual components than the result of any one factor or toxin. Recent studies have begun the process of "putting the bug back together" (91,92,152,209).

The classical approach to resolving the role of an individual molecule in a multifactorial system such as this disease process commonly involves two steps. The first step is to isolate and characterize the properties of a specific component. The second step involves creation of mutant strains with alterations in the molecule under study and investigations into what if any variation there is in the properties of the organism as a whole (213).

As in most Gram-negative organisms, *Bordetellae* endotoxin is the major structural component responsible for the integrity of the outer membrane. Its location and abundance presents the opportunity for association with most secreted and surface expressed molecules. This allows for possible interactions with all the previously mentioned virulence factors. Indeed, the very integrity of the bacterial cell wall is dependent on the numerous noncovalent bonds and linkages between endotoxin and the surrounding molecules. It is not unreasonable to suspect that the function of these molecules may also be affected by their presentation, in intimate proximity with the endotoxin, to the host. Certainly, outer membrane barrier functions are affected by alterations in endotoxin structure as has been established in a number of antibiotic susceptibility studies involving various enteric species (134,203). In the last decade the isolation and characterization of the endotoxins of *B. pertussis, B. parapertussis,* and *B. bronchiseptica* has been accomplished but their role in the intact cell has not yet been investigated. This is due, in part, to the fact that endotoxin is required by the cell for viability and thus, a direct deletion of the molecule is not possible. Creation of mutants such as Re, Rd, etc, however allow the functional consequences of LPS interactions to be analysed.

In my investigations I bypassed this problem by constructing, selecting and characterizing mutants with altered endotoxin. These studies represent the initial stages in elucidating the full immunological, biochemical and genetic nature of these mutations and the resultant implications concerning the role of *Bordetella* endotoxin in infection, disease and vaccine reactivity.

TABLE I.1: The Comparative Biological Activities of *Bordetellae* Endotoxin

(Watanabe et al., 208)

ASSAY	ORGANISM						
	Bordetella pertussis		**Bordetella parapertussis**		**Bordetella bronchiseptica**		**Enteric Control**
	Tohama 1	**AK-168**	**21815**	**AK-167**	**L3**	**H-214**	**E. coli O55:B5**
Pyrogenicity (Smallest dose to cause >0.6°C temperature increase in Japanese white rabbits)	10 ng/kg	100 ng/kg	100 ng/kg	100 ng/kg	1.0 ng/kg	10 ng/kg	10 ng/kg
Toxicity in Galactosamine-Sensitized Mice (Minimum dose required for lethality)	1.0 ng	1.0 ng	10 ng	10 ng	0.1 ng	1.0 ng	1.0 ng
Limulus Amoebocyte Lysate Activity (Minimum dose required to cause gelation)	0.01 ng/ml	0.1 ng/ml	0.1 ng/ml	0.1 ng/ml	0.01 ng/ml	0.1 ng/ml	0.1 ng/ml
Macrophage Activation[a] (at 1.0 μg/mouse dose)							
a) Phagocytosis of ^{51}Cr-labelled antibody-sensitized sheep RBCs	5.4%	4.4%	2.4%	2.0%	6.8%	5.3%	7.0%
b) Increase in cellular lysosomal enzyme (acid phospatase)	275 mg	260 mg	175 mg	160 mg	340 mg	260 mg	330 mg
c) Induction of cytostasis	56%	54%	18%	16%	63%	54%	56%
TNF Induction[a] (at 1.0 μg/mouse dose) (% inhibition of ^{3}H-thymidine uptake into L929 cells)	95%	85%	85%	95%	95%	99%	99%
Mitogenicity[a] (at 1.0 μg/ml dose) (^{3}H-thymidine uptake into C3H/HeN splenocytes (x 10^{-5}))	1.75 cpm	1.3 cpm	2.2 cpm	1.75 cpm	1.3 cpm	1.75 cpm	1.3 cpm

a) Values were approximated from graphic representation.

TABLE I.2: Biological Properties of *Bordetella pertussis* Lipooligosaccharide

ASSAY	RANGE OF ACTIVITY	CONTROL SPECIES	REFERENCE
TOXICITY			
Pyrogenicity[a]	2 - 100 ng/kg	2 - 10 ng/kg *E. coli / S. typhimurium*	11,28,107,208
Toxicity in galactosamine-sensitized mice[b]	0.35 µg/mouse	2.48 µg/mouse *E. coli*	11
Dermal Schwartzman assay[c]	8 - 14.5	control not detailed	11
Local inflammation[d]	7-20 mm	0-13 mm *C. parvum*	11
Mouse weight gain assay[e]	1.07 -3.4 µg	control not detailed	11
IMMUNOGENICITY			
Mitogenicity[f] **of 100µg/ml LOS on 5x10^{5} C3H/HeJ cells**	5000- 8000 cpm	2500 cpm *S. minnesota*	88
Endothelial cell adherence assay[g]	16 - 90 %	121 - 167 % *S. typhimurium*	1
Hepatic disfunction			
i)H.I. sleep-time index[h]			6,7
a)C3H/HeN mice	.45	.36	
b)C3H/HeJ mice	.54	.49 *E. coli*	
ii) Hepatic protein level[i]			6,7
a)C3H/HeN mice	.3	2.3	
b)C3H/HeJ mice	1.5	1.2 *E. coli*	
Adjuvant activity[j]			
i) *H. influenzae* A	2.4	3.5	11,28
ii) *H. influenzae* B	2.7	3.2 *C. parvum*	
Induction of nonspecific resistance to viral infections.[k]			
i) EMC	100%	25%	11,28
ii) SFV	100%	control not detailed	
Induction of nonspecific resistance to bacterial infections.[l]			
i) *Salmonella typhi*	100%	control not detailed	11,28
ii) *Staphylococcus aureus*	80%	control not detailed	
iii) *Pseudomonas aeruginosa*	100%	control not detailed	

a) Smallest dose to cause >0.6°C temperature increase in rabbits.

b) LD50 in mice.

c) Sum of six values of necroses (est. on a scale of 0 to 4) at the site of id. injection of LPS 6 hrs after iv injection of *Shigella dysenteriae* endotoxin into rabbits.

d) Diameter of zone of inflammation 20 hours after id. injection of LPS into rabbits.

e) Minimum dose of LPS below which no weight loss could be detected.

f) Measured by incorporation of [^{3}H]thymidine into dividing splenocytes.

g) Increase in relative adherence [(adherence with 1 µg/ml LPS/ adherence with TNF) x100]%

h) Hexobarbital-Induced sleep-time index: Mean sleep times 24 hours after iv injection of LPS
Mean sleep times 24 hours after iv injection of saline

I) Conc. of hepatic cytochrome P-450 (ng) in total microsomal protein (mg) 24 hrs after iv injection of LPS.

j) Humoral Response Index- increase in humoral antibodies 11 days after iv injection of LPS.

k) Mice surviving > 11 days after iv. injection of Encephalomyocarditis (EMC) or Semiliki Forest Virus (SMV).

l) Mice surviving a lethal dose of bacteria injected iv. 3 days after iv. injection of LPS.

FIGURE I.1

Structure of the *B. pertussis* LOS core with hypothesized differences in mutants due to Tn*5* gene disruption (7,17,18). Differences from enteric endotoxin include notable but presently unspecified increases in phosphorylation, a lack of extensive O-chain material and the presence of an unusually large number of amino sugars with non-acylated NH_2 groups.

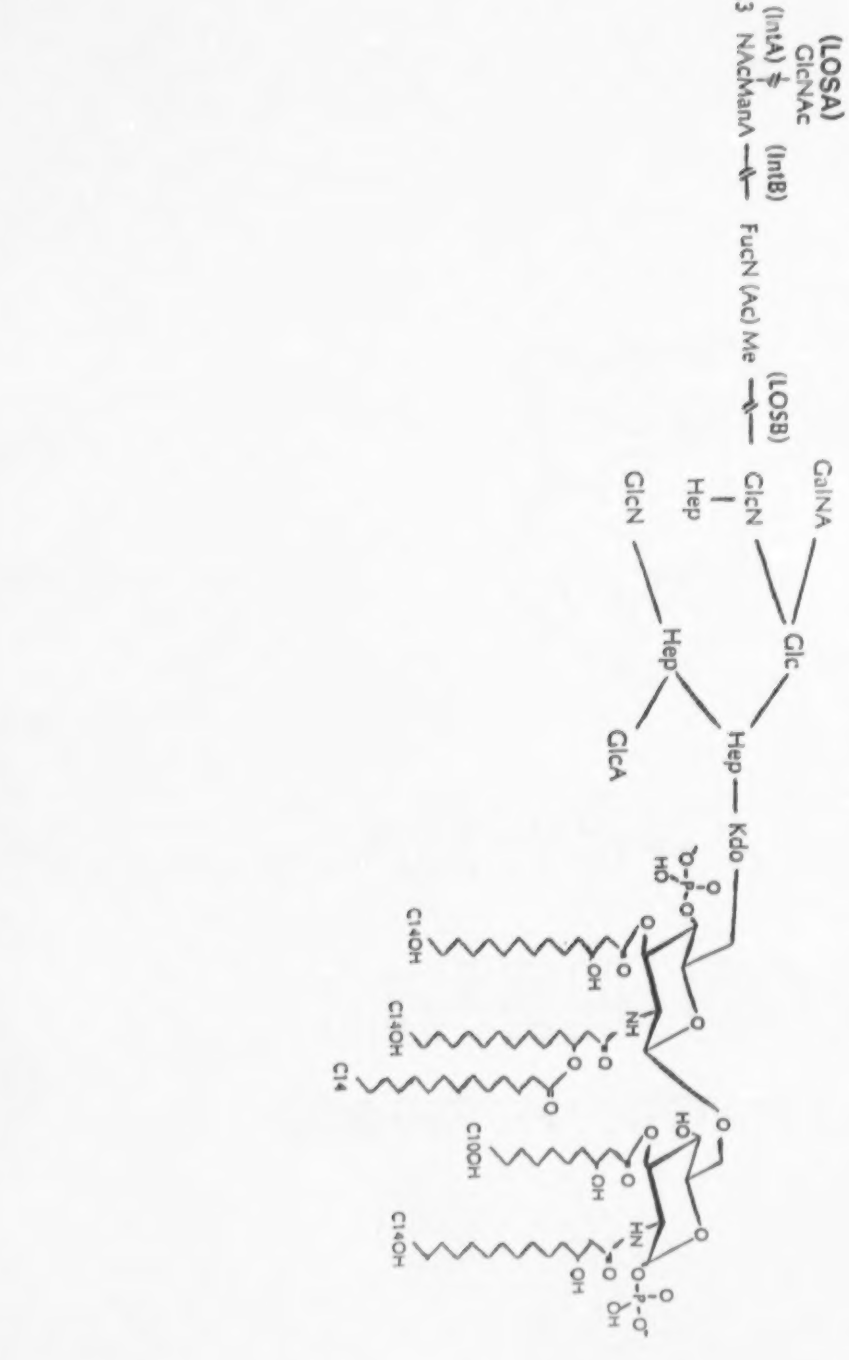
FucN(Ac)Me: N-acetyl-methyl fucosamine; GalNA: galactosaminuronic acid;

Glc: glucose; GlcA: glucuronic acid; GlcN: glucosamine;

GlcNAc: N-acetyl-glucosamine; Hep: heptose;

Kdo: 3-deoxy-D-*manno*-2-octulosonic acid;

2,3-NAcManA: 2,3-dideoxy-2,3-di-N-acetyl-mannuronic acid.


(LOSA)
GlcNAc
(IntA)
2,3 NAcManA
(IntB)
FucN (Ac) Me
(LOSB)
GalNA
GlcN
Hep
GlcN
Glc
Hep
Hep
GlcA
Kdo
C14OH
C14OH
C14
C10OH
C14OH
NH
HN
HO
OH

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CHAPTER II

Construction, Selection And Characterization Of *Bordetella parapertussis* Mutants With Tn*5*-Induced Alterations In Lipopolysaccharide.

A. INTRODUCTION

The *Bordetellae* are a genus of Gram-negative upper respiratory pathogens comprised of four species, *Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica* and *Bordetella avium* (32). *Bordetella pertussis* is the main causative agent of whooping cough. It possesses a large number of virulence toxins and factors, including pertussis toxin, dermonecrotic toxin, tracheal cytotoxin, filamentous hemagglutinin, pertactin and endotoxin (47). The clinical disease can also be caused by *B. parapertussis* infection but symptoms are routinely much milder (19,30). This has been hypothesized to be due, in part, to the lack of pertussis toxin, which, though genetically encoded, is not produced by *B. parapertussis* (35). *B. parapertussis* differs from *B. pertussis* in several ways; notably its faster growth rate (48 h versus 72 h for *B. pertussis*), negative oxidase reaction and tyrosinase activity (which leads to characteristic pigment production around colonies plated on blood agars) (32).

The exact scope and nature of *B. parapertussis* infection has been difficult to determine for a number of reasons. Clinical recognition of *B. parapertussis* infection has been thwarted by the mildness or absence of associated symptoms. Most reported data has been gathered from atypically severe clinical infections or as secondary observations in cases of *B. parapertussis* coinfection with *B. pertussis*

(12,22,27). Indeed, the frequency of co-isolation raises questions as to the exact epidemiological relationship between these two species. Another source of confusion is the possible laboratory misdiagnosis of *B. parapertussis* (13). In a large European study less than 20% of children infected with *B. parapertussis* presented with symptoms of clinical pertussis (8,22). Epidemiological data, however, indicate that *B. parapertussis* is a very widespread pathogen. Serological studies of several urban populations indicated an infection rate of more than 50% in the adult population, as evidenced by the possession of antibodies to *B. parapertussis* (24,43). Considering its possible scope, the incidence of misdiagnosis (as *B. pertussis*) and the high rate at which it is co-isolated with *B. pertussis* in clinical disease, it is very possible that *B. parapertussis* infection is of greater clinical significance than has been previously surmised.

Due to its historical identity as a minor pathogen, the elucidation of the contributions of the various toxins and factors of *B. parapertussis* to infection and disease has been slow, often only following in the path of similar studies with *B. pertussis.* Investigations have classically examined the putative roles of many of these molecules through the strategy of characterizing first the isolated molecule then the effect of genetic alteration (or deletion) of the molecule on the properties of the bacterial cell (20,25,45,48). Most of these factors are associated with the bacterial outer membrane which is the primary site of host/pathogen interaction. Endotoxin, as a major and integral component of the Gram-negative outer membrane plays an important part in the interactions between bacteria and host which characterize the processes of infection and immunity.

The toxic and immunological properties of isolated *B. parapertussis* endotoxin or lipopolysaccharide (LPS) have been characterized in several studies (23,44). *B. parapertussis* shares an chemically and immunologically identical O-side chain structure with *B. bronchiseptica*. It's core region, however, is quite dissimilar from that of the other *Bordetellae* (11). When compared to enteric species as well as other *Bordetellae,* the LPS of *B. parapertussis* exhibits a slight decrease in associated toxicity (ie. pyrogenicity, toxicity in galactosamine-sensitized mice) (23,44).

In an effort to better understand the role LPS plays in the function and integrity of the intact outer membrane and thus, bacterial pathogenicity I created and characterized six *B. parapertussis* Tn*5* insertion mutants with LPS alterations. Genetic analysis established the insertion of a single Tn*5* transposon in each mutant and indicated unique genomic sites for each Tn*5* insertion. Alterations in binding of the LPS mutants with bacteriophage and monoclonal antibodies reactive with the parent strain indicated structural or conformational disruption of outer membrane receptor functions and epitope exposure. The LPS mutants exhibited alterations in the complement-mediated bactericidal activity of the D13B11 mAb, a monoclonal antibody raised in our laboratory against the *B. parapertussis* O-chain which recognizes the the O-chain of *B. bronchiseptica* as well. In addition LPS mutants displayed conspicuous increases in sensitivity to human serum as well as several antibiotic and membrane-active agents. Overall, *B. parapertussis* strains with LPS alterations exhibited several notable disruptions in outer membrane barrier and receptor functions.

B. MATERIALS AND METHODS

Strains and monoclonal antibodies

Strains used in this study were *Bordetella parapertussis* strain 17903 which was obtained from J. Holweda (Michigan Department of Health, Detroit, Michigan, USA). *Bordetella pertussis* strain BP347, was obtained from A. Weiss (University of Cincinnati, Cincinnati, Ohio, USA). BP347 is the avirulent Tn*5* mutant which was produced by A. Weiss from BP338, which in turn was selected as a spontaneous nalidixic acid mutant of Tohama 1. The Tn*5*-containing vector pUW964, was also constructed and provided by A. Weiss (45).

The *B. parapertussis* parental strain YRD101 was selected as a nalidixic acid resistant strain of 17903. It was maintained on Bordet-Gengou Agar (BGA) with 60 µg/ml nalidixic acid while Tn*5*-insertion mutants were plated to BGA containing 60 µg/ml nalidixic acid and 25 µg/ml kanamycin. *E. coli* strain HB101 containing the Tn*5* plasmid, pUW964, was maintained on L-agar containing 25 µg/ml kanamycin (45).

B. pertussis and *B. bronchiseptica* 110H polyclonal antisera, LOSA specific monoclonal antibodies (mAbs), 14A8 and 1H2, as well as *B. parapertussis* O-chain specific mAbs, D13B11 and A7D12, were developed and characterized in our laboratory (Peppler et al., manuscript in preparation). Ascites fluid containing the mAbs were stored at -20°C, then thawed and diluted in blocking buffer (3% bovine serum albumin in 50mM tris-buffered saline, pH 7.0 (TBS)) for experiments.

B. parapertussis growth in broth and LPS extraction.

Initial 100 ml cultures of all *B. parapertussis* strains were inoculated from 1 day cultures harvested from BGA plates. These cultures were grown with aeration overnight at 37°C in Stainer-Scholte medium with 10 grams/L added casamino acids. The "seed" cultures were then used to inoculate 900 ml of the same medium and the broth was incubated for a further 3 days at 37°C with aeration. The LPS was extracted by the standard hot phenol/water method of Westphal and Jann commonly used for *B. parapertussis* LPS purification (31,49,50). Briefly, cultures were spun down by centrifugation at 5000 x g for 30 min at 4°C. After three washes with distilled water the pellet was dried, weighed then resuspended in a solution of 65°C 45% phenol and mixed for 15 min using a Sorval Omnimixer. The mixture was cooled to 10°C and centrifuged at 5000 x g for 50 min to produce a solution containing three distinct layers. The top aqueous layer was harvested and the remaining layers were re-extracted with 65°C water. The LPS was precipitated by incubating the pooled aqueous extracts with 1mg/ml sodium acetate and 3 volumes of acetone on ice overnight. The LPS pellet was collected by a 10 min centrifugation at 8000 x g in a refrigerated centrifuge. The LPS was then resuspended and washed three times in 70% acetone to remove small molecular weight contaminants and protein. After a final wash with 100% acetone the LPS was dried, weighed and resuspended by sonication to 3% weight/volume in distilled water. The mixture was then centrifuged at 80,000 x g for 6 h to remove residual RNA. The final pellet was dried, weighed and resuspended to a concentration of 100 µg/ml. Protein content was analyzed by Coomassie and silver stained SDS-PAGE gels.

Mutation of the wild-type *B. parapertussis* genome by random insertion of a Tn*5* transposon.

A nalidixic acid mutant of *B. parapertussis* strain 17903 was selected on BGA plates containing 60 µg/ml nalidixic acid. The nalidixic acid resistant variant was named YRD101 and was mutated by random insertion of a Tn*5* transposon. The transposon was transferred though conjugation of the parental YRD101 strain with an HB101 *E. coli* strain containing a Tn*5* suicide vector, pUW964 (45). *B. parapertussis* strain YRD101 was grown for 36 h on BGA containing 60µg/ml nalidixic acid. *E. coli* strain HB101 containing pUW964 was grown overnight on L-agar containing 25µg/ml of kanamycin. Each organism was swabbed from its growth plate (normal BGA) onto a mating plate (BGA containing either 10mM $MgCl_2$ or 10mM $MgSO_4$) and incubated at 37°C for 2.5 - 3.0 h (27). After incubation the organisms were swabbed from the mating plate to 2.0 ml of Stainer-Scholte broth. Mutants were selected by plating 100µl aliquots of a 10^{-1} dilution of this suspension onto BGA plates containing both 25µg/ml of kanamycin and 60µg/ml of nalidixic acid. This protocol routinely resulted in 100-300 kanamycin-resistant, nalidixic acid-resistant colonies per plate after 3 days growth at 37°C.

Western Colony Blot Analysis

LPS mutants were screened by western colony blot with the *B. parapertussis* O-chain specific monoclonal antibody D13B11. A round, sterile, 82.5 mm diameter, nitrocellulose disc (Bio-Rad) was soaked in TBS and pressed evenly onto each plate of mutants. The disc was then floated on chloroform in order to fix the organisms to the nitrocellulose. Unoccupied binding sites on the nitrocellulose were blocked by incubating the disc in blocking buffer (TBS with 3% bovine serum albumin (BSA) AND 1% skim milk powder) for 3 h at room temperature.

The primary antibody, D13B11, was added to the blocking buffer at a dilution of 1:100 and the blot was incubated for a further 2 h. The disc was then processed though a series of six 15 minute washes in TBS then incubated for 2 h in blocking buffer containing goat anti-mouse alkaline phosphatase conjugate (Sigma, St. Louis, MO). After an additional series of six 15 minute washes the blot was developed in nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as detailed by the manufacturer (Bio-Rad). Colonies which did not react with the anti-O-chain mAb, D13B11, were selected from the original plates, subcultured, and their LPS profiles confirmed by SDS-PAGE, silver stain, Western blot and whole cell ELISA analysis.

SDS-PAGE, Silver Stain, and Western Blot Analysis.

Lipopolysaccharide was visualized from *B. parapertussis* whole-cell lysates by solubilization of bacteria suspended in 50 mM Tris-glutamate saline at pH 7.5 (TGS) to an optical density of 0.3 measured at a wavelength of 540 nm. The method of Laemmli et al. was used (21,31).

Briefly, the organisms were centrifuged for 10 min at 10,000 x g, resuspended in 100 µl of Laemmli solubilization mixture and boiled for five min to disrupt the cells (21,31). Ten µg/ml of proteinase K was then added and the samples were incubated at 60°C for 2 h with intermittent vortexing. After cooling to room temperature, the LPS was precipitated by addition of 9 volumes of acetone and incubation on ice for one hour. After another 10,000 x g centrifugation for 10 min, the pellet was resuspended in 150 µl of Laemmli solubilization mixture and boiled for an additional 5 min. Samples were stored at 4°C until required.

The discontinuous system of Laemmli was used with a Tris-glycine buffer system (21). A Hoeffer SE 600 Series Vertical Slab Gel Unit was utilized to produce 14 cm wide by 16 cm long polyacrylamide gels. Ten µl of each sample was applied to the wells of an 18% (wt/vol. total) acrylamide gel (30:0.8, acrylamide : bis-acrylamide). The samples were stacked at 5 Watts then separated at 15 Watts constant power with cooling to 4°C. Prestained, low molecular weight markers from Gibco BRL (Burlington, Ontario, Canada) were utilized to follow the progress of each separation.

After proper separation had occurred (as indicated by the progress of the marker proteins and the dye front), the gels were either fixed in 40% ethanol and 5% acetic acid overnight, prior to silver stain, (by the method of Tsai and Frasch (40)) or transferred to 50 mM sodium phosphate buffer (pH 7.5) then assembled with a sheet of nitrocellulose slightly larger than the gel and loaded onto a Bio-Rad (Mississauga, Ontario, Canada) Trans-Blot apparatus according to the manufacturer's instructions. Transfer of the LPS from the gel to the nitrocellulose was completed after 3 h (as evidenced by prestained protein markers) utilizing the 50mM sodium phosphate buffer under a constant voltage of 27 volts (0.7 - 1.0 Amp) with cooling to 4°C.

The nitrocellulose membrane was transferred to a polypropylene dish and incubated at room temperature for 3 h in 3% BSA in TBS. The primary antibody (mAb D13B11) was added to the blocking buffer at a dilution of 1:100 and the blot was allowed to incubate for a further 2 h at room temperature. The membrane was then processed though a series of six 15 minute washes in TBS after which it was incubated for 2 h in 3% blocking buffer containing secondary goat anti-mouse antibodies conjugated with alkaline phosphatase (Sigma, St. Louis, MO) at a dilution of 1:5000. The blots were then developed with NBT/BCIP as described above for Western colony blots.

Whole Cell ELISA

Bacteria were suspended from BGA plates to an optical density of 0.20 at an absorbance of 540 nm (A_{540}) in PBS containing 0.1% Thimerosal. The bacteria were then killed by incubation at 56°C for 30 min Suspensions were used to coat 96- well plates by distributing 100µl of killed bacteria per well and incubating the plate for 18 h at 4°C. Plates were then centrifuged for 10 min at 2000 x g to further settle the bacteria onto the plate then washed three times with 0.8% saline (normal saline) containing 0.05% Tween-20 to remove unbound bacteria (with centrifugations for 10 min at 2000 x g between washes). The primary antibody was then diluted 1:100, added to wells, and plates were incubated for 2 h at 4°C. The plates were then put though three washes with normal saline-0.05% Tween-20, each followed by a 10 min centrifugation at 2000 x g. The alkaline phosphate conjugated secondary antibody was then added and plates were incubated at 37°C for 2 h, again washed and centrifuged three times then developed using the Sigma 104 phosphatase system (Sigma Chemical Co., St. Louis, MO). Attachment was qualitatively checked using Giemsa stain.

Southern Blot Analysis

To determine which bands contained the Tn*5* insertion, Southern hybridizations were done with a probe created by labelling a Tn*5* transposon with ^{32}P using the random primer labeling kit available from Gibco/BRL (Burlington, Ontario). Genomic DNA was isolated by the CTAB method (4). DNA was restricted for 2 h at 37°C with four separate restriction enzymes; *Bam*H1, *Cla*1, *Eco*R1, and *Sal*1. Digests were run on 0.6% agarose gels at 100 volts for 3 h and Southern analysis was carried out as detailed in Maniatis et al (36).

Briefly, the agarose gel was stained with ethidium bromide and photographed followed by depurination in 0.25 M HCl for 15 min to ensure adequate transfer of the DNA to the nylon membrane. The gel was then denatured and neutralized in preparation for transfer by the method of Southern et al (38). The DNA was transferred to Hybond N nylon membrane (Amersham, Oakville, Ontario, Canada) over 24 -36 h by capillary transfer (36,38).

The blot was removed with a forceps and allowed to air dry for one hour after which it was fixed under ultraviolet light for 5 min. The fixed blot was then incubated in prehybridization buffer (40% formamide, 30% 20 x SSC(Standard Sodium Citrate pH 7.0), 0.2% SDS, 10% 100 x Denhardt's Solution, and 0.5 mg/ml denatured salmon sperm DNA) for 2-4 h at 42°C (36). The denatured ^{32}P-labeled Tn*5* probe was added to the prehybrization mix and the blot was incubated in this hybridization solution overnight at 42°C. After hybridization, the blot was removed, rinsed in 2 x SSC, washed for 1 hour at 42°C in 2 x SSC then wrapped in plastic wrap and exposed to X-OMAT AR autoradiograph film (Kodak, Toronto, Ontario, Canada) for 1-3 h at -70°C.

Radial Diffusion Immunoassay for Assaying Bactericidal Activity and Sensitivity to Human Serum.

The method of Fernandez and Weiss was used (personal communication). Briefly, ten ml of 1% agarose in Stainer-Scholte broth part "A" was autoclaved then cooled to 50°C. To this medium 0.1 ml of 100x Stainer-Scholte broth part "B" and 0.25 ml of 6% BSA (in Stainer-Scholte broth (SSB)) was added. Media were left in 50°C water bath until use. Bacteria were harvested from BGA plates into SSB to a final OD_{660} of 0.2. Two hundred µl of the bacterial solution was then added to the Stainer-Scholte agarose (SSA) media, vortexed then immediately poured into a square Integrid petri dish. The plate was then left to harden on a level surface at room temperature for 10 min.

Using the grid marks of the plate a 3 mm Ouchterlony-type aspirator punch was then used to punch 25 equally distributed wells in each agarose plate. All agarose and liquid in the wells was carefully aspirated off. For determination of human serum sensitivity, 5 µl of fresh, pooled human serum was added to each well. For determination of the bactericidal activity of the D13B11 mAb to *B. parapertussis* strains 5 µl of 50% guinea pig serum/50% mAb D13B11 was added to each well. Guinea pig sera was originally diluted in SSB to a level (1:4) where complement could not, alone, lyse *B. parapertussis* cells. All assays were done in pentuplicate; that is 5 individual wells were tested for each sample for each of the 4 independent experiments.

Plates were incubated at room temperature for 3 h to allow serum to diffuse. A 10 ml overlay of SSA was then added, the agarose was allowed to harden and the plates were incubated for 24 to 48 h at 37°C. Zones of inhibition were measured with the use of a Bausch and Lomb stereomicroscope fitted with an ocular micrometer.

Antibiotic Sensitivity Assay

Strains were grown for 36 h on BGA then suspended in 4 ml of SSB to an optical density of 0.20 at a wavelength of 540 nm. The suspension was then diluted and plated in duplicate to BGA plates to determine the total viable count of organisms used in the inoculum. The agents were selected based on previous studies on the effects of various antibiotics on *Bordetellae* species and enteric LPS (5,6,15,28,39).

Twofold serial dilutions of each antibiotic were performed in triplicate in 96-well tissue culture plates from a stock solution which was predetermined using the parental strain, YRD101, as a control reference strain. Control wells with the antibiotic diluent alone, were included in each assay. 100 µl of the 10^{-1} dilution of bacteria was added to the final 100 µl volume of each antibiotic or control (diluent alone) well. The plates were incubated for 3 days at 37°C. Minimum Inhibitory Concentration (MIC) was measured as the last clear well (no turbidity) both by eye and by ELISA reader at an absorbance of 540 nm. Final MIC data was based on geometric mean titres.

Phage sensitivity and monoclonal antibody plaque inhibition assays.

Sensitivity to bacteriophage which commonly lyse the parental strain YRD101 was assayed in the following manner. After initial isolation from *B. bronchiseptica* (L1) and *B. pertussis* bacteriophage were propagated in tryptic soy broth (TSB). *B. parapertussis* strains grown on BGA for 24 h at 37°C were suspended in TSB to 0.2 at OD_{540}. 0.4 ml of the bacterial suspension was added to 2.0 ml of 0.8% agarose and plated. In plaque inhibition studies 1 ml of a 1:100 dilution of ascites containing an O-chain specific mAb, D13B11, was also added. After 2 h, 10 µl of phage (approximately 10^6 phage) were spotted onto the agar in triplicate. The plates were allowed to dry with the lids ajar for 30 min. The plates were incubated at 4°C for 30 min then transferred to 37°C. Presence or lack of plaques on the bacterial lawn was assessed after 18 h. Three independent experiments were performed with samples in triplicate.

C. RESULTS

General characteristics of LPS mutants.

All LPS mutant strains demonstrated a colonial morphology which was phenotypically similar to the parent strain, YRD101. That is, all mutant strain colonies were hemolytic on BGA and produced characteristic browning of the media. In broth culture and on clear agar the development of a pink to rust colour was characteristic of both parental and mutant strains within three days of culture. Growth rate on agar, likewise, was unaffected by the presence of the Tn*5* insert. The mutant colonies like the parental strain, YRD101, all formed colonies of approximately 1 mm in diameter in approximately two days and the presence of hemolytic activity indicated a retention of parental virulence.

Tn*5* mutants with altered LPS were negatively selected from the mutant population (nalidixic acid and kanamycin - resistant colonies) using D13B11, a monoclonal antibody which recognizes a presently undetermined epitope on the O-chains of both *B. parapertussis* and *B. bronchiseptica*. A lack of reactivity of colonies to this mAb indicated a modification of the LPS O-chain. The LPS was isolated from such colonies and further analysed by SDS-PAGE. Coomassie and silver stained SDS-PAGE gels of whole cell lysates indicated no notable alteration in protein content in any of the selected mutants.

Analysis of phenotypic alterations in the profiles of the isolated LPS of Tn*5* mutants.

Mobility in SDS-PAGE / Western Blot Analysis.

Seventy-six mutants with detectable alterations in LPS phenotype were initially selected after Tn*5* insertion by the negative reaction of colony blots with the *B. parapertussis* O-chain mAb D13B11. An absence of D13B11 binding to the colony indicated an absence or alteration of the D13B11 O-chain epitope recognized by the mAb and therefore an alteration in LPS structure. Each colony with a putative LPS mutation was further analyzed by SDS-PAGE, silver stain and western blot analysis with the *B. parapertussis* O-chain mAbs D13B11 and A7D12 (which reacts with a "deeper", i.e. lower molecular weight, O-chain epitope) (Peppler et al., manuscript in preparation). Of the 76 LPS mutants initially isolated these data concurrently indicated the presence of 3 phenotypic subsets of which there were several different types (Table II.1, Figure II.1) :

1) Normal Core, slightly altered O-chain. (Bpp-43)

The LPS of Bpp-43 was the most similar to the parental strain, YRD101 in both silver-stained SDS-PAGE gel analysis and reactivity to D13B11 in Western blots (Figure II.1A, lane 3). An alteration in the presentation of LPS O-chain sugars was indicated, however, by the great decrease in reactivity of the LPS to D13B11 when presented on the whole cell in colony blot and whole cell ELISA assays. Bpp-43 also had the only isolated LPS which reacted well with *B. bronchiseptica* and *B. pertussis* polyclonal antisera; that is to a similar degree as the parent strain (Table II.1, Figure II.1B, lane 3).

2) Normal Core, deeply altered O-chain. (Bpp-54, Bpp-68)

Although the core appeared unchanged from the LPS of the parental strain, YRD101, SDS-PAGE indicated a complete truncation of the O-side chain moiety in both Bpp-54 and Bpp-68 (Figure II.1A, lanes 4 and 5). Reaction of the isolated LPS with two O-chain specific mAbs, D13B11 and A7D12, on heavily loaded Western blots, however, indicated the presence of a notable, albeit greatly abridged, O-chain moiety (Figure II.1B, lanes 4 and 5). Smaller amounts of LPS resulted in a great reduction in the band of Bpp-68 and the total loss of the band for Bpp-54 (data not shown). While the Bpp-68 LPS O-chain was comprised of a narrow, band with relative mobility (M_r) of approximately 14,000-15,000 Da (as estimated by prestained protein molecular weight markers), the altered O-chain of Bpp-54 was composed of a more diffuse band of slightly higher molecular weight material, approximate M_r of 15,000-20,000 Da . The A7D12 mAb reacted with this isolated material better than did D13B11. The decrease in amount and alteration in reactivity of the O-chain material of Bpp-54 and Bpp-68 corresponds with the weak reactivity of the strains in whole cell assays with the O-chain mAbs, D13B11 and A7D12 (Table II.1).

3) Altered Core, deeply altered O-chain. (Bpp-35, Bpp-37, Bpp-76)

Bpp-35, Bpp-37 and Bpp-76 all completely lacked O-chain structures as visualized though mobility of the LPS on an 18% SDS-PAGE gel (Figure II.1A). Bpp-76 also showed no reactivity with the O-chain specific mAbs in assays of either whole cell bound (whole cell ELISA, colony blot) or isolated LPS (western blot) (Table II.1, Figure II.1B). Bpp-35 and Bpp-37 displayed some reactivity to A7D12 (Figure II.1B, lanes 1 and 2). In very heavily loaded blots 2 bands were seen; a very faint, thin band of approximate M_r of 18,000 Da, and a wider band of slightly lower molecular weight. Unlike the natural ladder pattern observed in the parental strain, YRD101 as well as the mutant strains, Bpp-43, Bpp-54, and Bpp-68 the two discrete band regions in Bpp-35 and Bpp-37 did not appear to have intermediate sized bands between them. It is not known whether these poorly reactive bands are due to decreased reactivity of the LPS molecule in the band or decreased concentration in comparison with the strongly reactive bands found in Bpp-43 and YRD101.

As no core-reactive mAbs were available for this study, further characterization was determined by mobility on SDS-PAGE gels (Figure II.1A). The cores of each of these three mutants, Bpp-35, Bpp-37, and Bpp-76, were all different from that of YRD101. Bpp-76 had a core structure which appeared to be of notably higher relative mobility than that seen in the parent strain (Figure II.1A, lanes 1 and 2). Bpp-35 and Bpp-37 each had distinct additional bands of slightly higher molecular weight than did YRD101. Bpp-37 had what might be considered either a slightly structurally modified core band or a very truncated O-chain in the designation of the minor chain of bands which comprised this additional region (just above 6000 Da protein marker). Bpp-35 had a lower molecular weight band and in addition to this possible core component the gels of the first several cultures indicated the presence of an additional band that was lower in molecular weight than the YRD101 band. This band has become less discrete upon repeated subculture and even fresh cultures from the -70°C freezer do not display this band well. It can only be speculated that the appearance of the very low molecular weight band in Bpp-35 LPS is dependent on certain culture conditions which we have not been able to duplicate. This would correspond with the dependence noted in our laboratory of O-side chain production in YRD101 on culture conditions, notably age of harvest (unpublished observation).

Growth and Extraction of LPS from Control and Mutant Strains.

All mutants were grown in one litre broth culture and the LPS extracted by the standard hot phenol/ water protocol used for extraction of LPS from the parental strain , YRD101 (Table II.2). The LPS of those mutants lacking an extensive O-chain moiety extracted very poorly with this method. Although similar in bacterial growth, the yields of Bpp-35, Bpp-37, and Bpp-76 LPS were 15%, 26% and 52%, respectively, of that of YRD101. LPS yields of Bpp-43, Bpp-54, and Bpp-68 were all within 75% of the YRD101 yield weight. The extraction procedure was repeated twice with similar results. Results were then averaged.

Genetic analysis of Tn*5* insertion in genomic DNA of LPS mutant strains.

Southern Blot Analysis

The presence of a single Tn*5* insert in each of the mutant strains was confirmed by Southern analysis (Figure II.2). The Tn*5* probe hybridized with a single band in mutant and control strains when DNA was digested with enzymes such as *Cla*1 and *Eco*R1 which do not recognize sites within the Tn*5* gene. When digested with enzymes such as *Bam*H1 and *Sal*1 which have a single cut site within the Tn*5* gene each DNA digest produced two bands which hybridized with the Tn*5* probe.

Analysis of LPS Alteration on Bacterial Outer Membrane Function

Alterations in Sensitivity of LPS Mutants to Lysis by *B. parapertussis*-specific Bacteriophage.

Each mutant strain was assayed for sensitivity to two bacteriophages which are known to lyse the parental strain YRD101, and for which evidence suggests that the LPS plays an important role in receptor functions (Table II.1). One of these lysogenic phage, L1, is native to *B. bronchiseptica* while the second phage was originally isolated from *B. pertussis*. Neither phage is known to lyse either *B. pertussis* or *B. bronchiseptica* but both have been shown to effectively lyse *B. parapertussis*.

Only Bpp-43 was affected by either bacteriophage. The plaques formed in the same amount of time and were similar in size to those produced on lawns of YRD101. As well, experiments where D13B11 was used to compete for LPS receptor sites abolished bacterial lysis and plaque formation in both YRD101 and Bpp-43.

Alterations in Bactericidal Activity of D13B11 mAb in the Presence of Guinea Pig Complement.

The bactericidal activity of D13B11 against YRD101 had previously been established using the broth dilution/plate count protocol described in chapter IV (M.S. Peppler, manuscript in preparation). The bactericidal activity of D13B11 and guinea pig complement against each mutant strain as well as the parent strain, YRD101, was subsequently assayed using a radial diffusion bactericidal assay (Figure II.3). Guinea pig sera used in the assay was diluted in SSB to a level (1:4) where complement did not, alone, lyse *B. parapertussis* cells. While YRD101 and Bpp-43 were lysed by activation of the classical complement pathway those mutants with greater truncations in their O-chains were less sensitive to this killing. Lytic zones on lawns of Bpp-54, Bpp-68 and Bpp-76 were all reduced by approximately 28 percent in comparison with the parent strain. Mutants with the smallest molecular weight LPS, Bpp-35 and Bpp-37, were approximately 43 percent less sensitive to the bactericidal activity of D13B11.

Alterations in Sensitivity of LPS Mutant Strains to Pooled Human Serum.

The sensitivity of *B. parapertussis* mutants and YRD101 to pooled normal human sera was assayed by a radial immunodiffusion assay similar to the protocol utilized to determine bactericidal activity of D13B11 (Figure II.4). Both YRD101 and Bpp-43 were found to be 31 to 38 percent more sensitive to the complement-mediated killing activity of human sera than those mutants with altered LPS; Bpp-35, Bpp-37, Bpp-54, Bpp-68, and Bpp-76.

Alterations in Sensitivity of LPS Mutants to Antibiotics and Membrane-Active Agents.

The sensitivity of mutant *B. parapertussis* strains with altered LPS to twenty three different antibiotics, detergents and chelators was compared with that of the parental strain, YRD101 in broth dilution assays (Tables III.3 and III.4).

As all Tn*5* mutants were initially screened based on the acquisition of kanamycin resistance it was not surprising to find that they all possessed marked reductions in sensitivity to kanamycin and the structurally related aminoglycoside, amikacin. The decreased sensitivity to streptomycin has been attributed to the presence of the Tn5 transposon but no definate mechanism has been established (45).

None of the LPS mutants differed considerably from the parental strain, YRD101, in their susceptibility to nalidixic acid, chloramphenicol, erythromycin, gentamicin, oleic acid, polymyxin B, or EDTA. A notable increase in sensitivity for all mutants, that is a difference of 2 or more dilution factors, was observed with only one antibiotic, novobiocin. A slight increase in susceptibility of the mutant strains (a consistent difference of 1 dilution factor over 2 triplicate assays) to cephalothin, imipenem, sulphamethoxazole, and EGTA was also noted. Mutants also exhibited a slight increase in sensitivity to rifampicin.

Individually, Bpp-35 displayed the most marked differences in susceptibility to the agents assayed when compared with the parent strain. Bpp-35 was found to be 4 fold more sensitive to hydrophilic antibiotics such as ampicillin and cephalothin, 8 fold more susceptible to cefotaxime and over 10 fold more susceptible to imipenem. Bpp-35 was also 6 fold more sensitive to the hydrophobic antibiotic, novobiocin, and 4 fold more susceptible to the large peptide antibiotic vancomycin and the Ca^+ specific chelator, EGTA.

Bpp-37 showed an altered sensitivity to only 2 agents; a 6 fold increase in sensitivity to novobiocin and a 4 fold decrease in rifampicin susceptibility.

Bpp-43 exhibited increases in susceptibility to hydrophobic antibiotics such as fusidic acid (4 fold) and novobiocin (6 fold) as well as hydrophilic antibiotics such as cefotaxime (16 fold), and the large peptide antibiotic, vancomycin (2.5 fold).

Bpp-54 was 4 fold more sensitive to cefotaxime as well as cephalothin than YRD101 and 4 fold more susceptible to killing by imipenem.

Bpp-68 was also more sensitive to cephalothin (4 fold) and imipenem (3 fold). It was also the only mutant to exhibit a marked increase in sensitivity to trimethoprim (4 fold).

Of all the mutant strains, Bpp-76 was most like the parent strain, YRD101, in its susceptibility to the various agents assayed. An increased sensitivity was notable only in the case of novobiocin where Bpp-76 was found to be 6 fold more sensitive than YRD101.

D. DISCUSSION

Several studies have examined the properties of the isolated LPS of *Bordetella parapertussis* (0,23,44). The role of this molecule in the multifactorial process of clinical disease (whooping cough), however, has yet to be elucidated. As a major component of the extracellular leaflet of the outer membrane the lipopolysaccharide has the opportunity and structural properties necessary for interaction with numerous extracellular and cell-associated molecules (17,29).

I began my investigations into the nature and consequences of these possible interactions by constructing a number of *B. parapertussis* mutants with altered LPS moieties. Mutant colonies were initially screened by lack of reactivity with an LPS-specific monoclonal antibody, D13B11. Six mutants were selected from this primary pool on the basis of structural differences notable in the SDS-PAGE profiles of their isolated LPS. Further physical characterization of the mutant LPS was endeavored with the use of various mAbs reactive to different *Bordetellae* LPS/LOS epitopes. Polyclonal antisera of *B. bronchiseptica* as well as the *B. parapertussis*-specific mAbs, D13B11 and A7D12, bound strongly to the isolated LPS of Bpp-43, considerably less so with Bpp-68 LPS and largely unreactive with the LPS of Bpp-35, Bpp-37, Bpp-54 and Bpp-76.

These results differed slightly from the reaction of these mAbs to the LPS as presented on the whole cell. Colony blots and whole cell ELISAs showed a significant decrease in the reactivity of Bpp-43 with the *B. parapertussis* mAbs when compared with the parental strain, YRD101. This suggested that the binding site for D13B11 was present on the LPS molecule however its exposure in the whole cell had been altered. As the SDS-PAGE profile of Bpp-43 did not differ noticeably from that of the parental phenotype the possibility must be entertained that the Tn*5* insertion may have affected a spatially associated protein rather than the LPS, itself. Several studies have established the importance of protein/LPS associations in the outer membrane. Changes in LPS structure have been demonstrated to alter protein receptor function for phage and disrupt binding of mAbs which recognize the LPS/protein (OmpF, OmpC porin) complex (18,42).

Overall, athough Bpp-43 differed from the parental phenotype only in decreased reactivity of the whole cell to D13B11, all five of the remaining mutants were notably different structurally and antigenically. As well, southern blots of several restriction digests indicated unique insertion sites for each mutant. Thus, the phenotypic changes noted in the different mutants appear to be the result of the disruption of different genes.

After establishing the uniqueness of each mutant, investigations into the consequence of each LPS alteration on cell function were initiated. Although all mutants grew at rates similar to the parental phenotype, the extractability of the LPS with the standard hot phenol/water protocol decreased as LPS truncation increased. Mutants such as Bpp-35, Bpp-37 and Bpp-76 yielded almost no LPS. This is representative of the significant differences in chemical properties which have been noted in many prior studies to result from structural alteration of the amphoteric LPS molecule (16). While the hot phenol/water method is useful for extracting hydrophilic endotoxins from the whole cell, a different protocol has been developed for extraction of less hydrophilic "rough" endotoxins which lack an extensive polysaccharide O-unit (16,49,50). In addition, the *B. pertussis* and *B. bronchiseptica* phages reacted with Bpp-43 alone, indicating changes in receptor functions of the membrane. Thus, although the exposure of the LPS in Bpp-43 was altered enough to modify its ability to react with the D13B11 mAb the changes did not affect its ability to bind either of the two phages. Likewise, the bactericidal effect of D13B11 was unaffected. It appears that the exposure of the D13B11 epitope on an organism in a fixed colony (western colony blot) or on a solid matrix (whole cell ELISA) differs from that of the organism in agar plates.

LPS is a proven activator of complement-mediated bactericidal activity and antibody-dependent complement killing has long been acknowledged as an important clearance mechanism in diseases caused by gram-negative bacteria (1,9,26,33). Thus, the effect of LPS modification on bactericidal activity was assayed as an important first test of the effect of LPS alteration on whole cell function. Bactericidal assays with the different LPS mutants showed a decrease in susceptibility to D13B11-mediated bactericidal activity as LPS truncation increased. It is probable that the decrease in bactericidal activity was the result of alterations in the D13B11 binding site as the result of O-chain truncations.

To expand on the results obtained from the bactericidal assay it was decided to investigate the effect of the LPS alterations on bacterial sensitivity to killing by whole human sera. Previous studies noted the increased resistance to killing by human sera of smooth *Bordetellae* strains as opposed to rough (bacteria lacking O-chain polysaccharide) (10). LPS acted as a protective barrier and truncation exposed sites vulnerable to the complement attack complex. Studies with the *B. parapertussis* LPS mutants and whole sera showed a reciprocal response to LPS truncation as to what was found in laboratory bactericidal assays. A clear increase in susceptibility to killing by human sera was observed as LPS truncation increased. This increased sensitivity may be due to alterations in the overall barrier functions of the LPS or the binding sites responsible for bacterial killing by whole human sera reside in core-associated sugars rather than exclusively in the more exposed sugars of the O-chain which D13B11 recognizes.

Other studies by Fernandez and Weiss recently documented the increased resistance of virulent *Bordetellae* to complement-mediated killing by human serum (14). This mechanism is mediated by the *brk* gene through two proteins, BrkA and BrkB, and under the control of the *bvg* locus. As this susceptibility is abolished by the addition of 10mM EGTA and $MgCl_2$, it has been postulated that complement killing occurs via the antibody-dependent, classical pathway. Although all three species contain the *brk* gene it is notable that *B. parapertussis* alone does not express the Brk proteins (A. Weiss, personal communication). Thus, the differences in serum sensitivity and D13B11-mediated bactericidal activity found in the *B. parapertussis* LPS mutants are not related to the activity of the Brk system.

The functional consequences of LPS alteration on the cell were further investigated though examination of the response of the different mutants to several antibiotics and membrane-active agents. Unlike the results obtained with human sera and the bactericidal monoclonal antibody, D13B11, the extent of LPS truncation did not directly correlate with changes in antibiotic sensitivity. This contraindicates the involvement of a lost or altered antibiotic binding site and implies a more indirect route by which the LPS alterations affected the activity. The variation in susceptibility to diverse antibiotics was unrelated to the extent of LPS truncation. This supports the theory that alterations in the LPS result in alterations in the interactive forces which stabilize the outer membrane. It is these disruptions in the membrane which result in increased antibiotic sensitivity rather than the altered LPS, itself.

Of the many antibiotics assayed only novobiocin exhibited a significant increase in toxicity towards all of the LPS mutants when compared with the parental strain, YRD101. Hydrophobic antibiotics such as novobiocin had been previously documented to exert enhanced activity on *Salmomella typhimurium* and *E. coli strains* with LPS alterations. Although many avenues of speculation have been offered the most accepted theory suggests that this enhancement is the consequence of the increase in phospholipid concentration in the outer leaflet as a means of compensating for the loss of endotoxin-associated proteins. (17,28,29,39). The drug passes though the membrane to its intracellular active site with much greater speed and ease than would normally occur with an "intact" outer leaflet composed of tight LPS/LPS interactions. That even the very mildly truncated Bpp-43 was equally affected indicates that the aspect of LPS alteration important to this susceptibility may lie less in the actual structural truncation and more in LPS interaction with associated molecules (LPS/LPS, LPS/protein, etc.). It is possible that the novobiocin, alone (of all the hydrophobic antibiotics tested), was able to exploit whatever disruption occurred in the membrane as the result of even the most minor of the structural alterations, that found in Bpp-43, and reach the hydrophobic interior with greater ease. This is supported by previous studies with enteric species where alteration in LPS structure most drastically affected novobiocin sensitivity of the host of antibiotics tested (34,37).

Many of the mutants also exhibited some increased sensitivity to hydrophilic antibiotics. Again, the mutants differed greatly in susceptibility to these antibiotics irrespective of the degree of LPS structural truncation. Although the core-minus mutant Bpp-35 showed the greatest sensitivity to the largest number of hydrophilic antibiotics, the second most sensitive strain was Bpp-43. The LPS of this strain is so structurally and immunologically similar to the parent strain that it is possible that the mutation has affected an accessory group such as a phosphate rather than the LPS sugar structure. It is also possible that the mutation is in an associated protein which in turn causes a conformational alteration in the LPS presentation. Hydrophilic antibiotics usually pass though the membrane though pores (18,29,42). Changes in sensitivity due to LPS is theorized to occur by altering the charge of surrounding molecules which, in turn, either changes the size of the pore or the attraction of the pore interior to the antibiotic. Several studies have documented the ability of various outer membrane components, such as adenylate cyclase toxin to cause membrane disruption and pore formation in *Bordetella pertussis* (3,7). An alteration in LPS structure could disrupt the interaction of LPS with one or several of these molecules.

The LPS of *B. parapertussis*, like the LPS of most bacteria, carries a negative charge due to the presence of attached phosphate groups on the O-chain, core, and lipid A moieties. The O-chain of *B. parapertussis* is a unique, linear, unbranched homopolymer of approximately 20 residues of 1,4-linked 2,3-diacetamido-2,3-dideoxy-α-L-galactopyranosyluronic acid (11). Thus, as well as the negative charges resulting from the phosphate groups, the amido groups of the O-chain contribute positive charges to the LPS molecule. These charged groups play an important role in the LPS interactions which stabilize the outer membrane (17,29,42) Divalent cations and cationic detergents such as polymyxin B destabilize these interactions by displacing the normal positive ions and/or decreasing the normal surface potential by reducing the overall negative charge of the membrane. The result of such disruptions can range from a total lysis of the cell (as is produced by treatment with polymyxin B) to the creation of a "leaky" membrane such as is produced in the creation of competent cells by $CaCl_2$ or $RuCl_2$ treatment (29,42). Chelating agents such as EDTA and EGTA remove cations and the resulting charge repulsion amongst the LPS molecules creates a "leaky" membrane.

A notable difference in sensitivity was observed in Bpp-35 to EGTA, which preferentially removes Ca^{++} ions. This indicates that Tn*5* insertion has caused a conformational or structural-based disruption of the surface potential. As the actual structural alterations in the LPS mutants under investigation are not yet clear it can not be stated how drastic a change in charge the altered LPS may have undergone nor can the exact nature of any change be confirmed. Phenol/water extraction data indicates a substantial decrease in hydrophilicity in the most truncated mutant LPS strains; Bpp-35, Bpp-37, and Bpp-76, which may be related to a loss of charge potential. More detailed chemical analysis of the truncated LPS types found in these mutants , however, is required in order to clarify the exact nature of each antibiotic interaction. Any possible heterogeneity of this LPS could greatly complicate these studies.

The investigations described in this document represent the initial studies into the nature and consequences of LPS interactions with other molecules within the outer membrane of B. *parapertussis*. Extensive in vitro and in vivo studies with the LPS mutants (i.e. cellular invasion assays, the mouse weight gain assay, rabbit pyrogenicity assay, chick lethality assay, etc.) would help elucidate the full role of LPS in *B. parapertussis* infection and disease. This cause could be equally served in utilizing the LPS mutants as background organisms for a second specified mutation; i.e. other virulence factors such as the Brk proteins, filamentous hemagglutinin, adenylate cyclase toxin. In addition, these mutants represent the first opportunity to chemically and genetically characterize the *B. parapertussis* LPS biosynthetic pathway. A better understanding of the complex interactions of LPS with LPS as well as other molecules in the outer membrane would help unravel the complicated and intricate mechanisms by which *B. parapertussis* initiates infection and causes disease. Such examination of outer membrane functions might also provide insight into the etiological and clinical relationship between *B. parapertussis* and *B. pertussis.*

TABLE II.1: Phenotypic Characterization Of *Bordetella parapertussis* Mutants

STRAIN	SDS-PAGE	WESTERN BLOT ANALYSIS[a]					COLONY BLOT[b]	WHOLE CELL ELISA[c]				SENSITIVITY TO BACTERIOPHAGE LYSIS[d]	
		D13B11 (Bpp-O-chain mAb)	A7D12 (Bpp O-chain mAb)	14A8, 1H2, BL2 (BpLOSA mAbs)	polyT1 (Bp pAbs)	110H+ (Bbs pAbs)	D13B11 (Bpp-O-chain mAb)	D13B11 (Bpp-O-chain mAb)	A7D12 (Bpp O-chain mAb)	polyT1 (Bp pAbs)	110H+ (Bbs pAbs)	L1 (Bbs Phage)	(Bp Phage)
YRD101	O+	O+	O+	-	+	+	++	++	++	++	++	+	+
Group 1													
Bpp- 43	O+	O+	O+	-	+/-	+	-	-	+/-	++	++	+	+
Group 2													
Bpp- 54	O-/+	O-/+	O-/+	-	-	-	-	-	-	+	+	-	-
Bpp- 68	O-/+	O+/-	O+/-	-	-	-	-	-	-	+	+	-	-
Group 3													
Bpp- 35	O-,core+	-	+/-	-	-	-	-	-	-	+	+	-	-
Bpp- 37	O-,core+	-	+/-	-	-	-	-	-	-	+	+	-	-
Bpp- 76	O-, high core	-	-	-	-	-	-	-	-	+	+	-	-

a,b) Scored visually - with "++" being positive control strain against which antibody was originally raised (i.e. original inoculum), "+/-" being less bands, "-/+" being faint, fewer bands, and "-" being "no antigen" negative control.
c) Scored by ELISA reader - with "++" being positive control strain against which antibody was originally raised (i.e. original inoculum), "+/-" being less bands, "-/+" being faint, fewer bands, "-" being "no antigen" negative control.
d) Scored by lack (-) or production (+) of plaques on bacterial lawn.

TABLE II.2: Growth and LPS Extraction of *Bordetella parapertussis* LPS Mutant and Control Strains

STRAIN OF *BORDETELLA PARAPERTUSSIS*	PHENOTYPE (SDS-PAGE, WESTERN BLOT)	BACTERIAL YIELD IN G (WET WEIGHT / L CULTURE)	LPS YIELD IN G (DRY WEIGHT/ L CULTURE)	% WEIGHT IN COMPARISON WITH YRD101 (parent strain)
YRD101	O+	9.38	0.019	100.0 %
Group 1				
Bpp- 43	O+/-,	10.34	0.016	84.21 %
Group 2				
Bpp- 54	O-/+	10.03	0.019	100.0 %
Bpp- 68	O-	9.743	0.015	78.95 %
Group 3				
Bpp- 35	O-	8.996	0.003	15.79 %
Bpp- 37	O-	8.603	0.005	26.32 %
Bpp- 76	O-, high core	9.807	0.010	52.63 %

TABLE II.3: Notable[a] Alterations in Antibiotic Sensitivity Of *Bordetella parapertussis* LPS Mutant Strains

BACTERIAL STRAINS	ANTIBIOTIC SENSITIVITY (MIC in µg/ml)										
	Increase in Sensitivity					Decrease in Sensitivity	Tn5-Related Decrease in Sensitivity		Mixed Alterations in Sensitivity		
	Cephalothin	EGTA	Imipenem	Novobiocin	Vancomycin	Rifampicin	Amikacin	Kanamycin	Ampicillin	Cefotaxime	Fusidic Acid
Control Strain YRD101	20.0	2.5 mM	5.00	0.90	320	1.25	6.00	8.00	32.0	10.0	5.00
Mutant Strains											
Group 1											
Bpp-43	10.0	1.25 mM	2.50	0.30	120	2.50	>96.0	>96.0	16.0	0.63	1.25
Group 2											
Bpp-54	5.00	1.25 mM	1.25	0.30	160	2.50	>96.0	>96.0	16.0	2.50	2.50
Bpp-68	5.00	1.25 mM	2.50	0.30	160	2.50	>96.0	>96.0	32.0	20.0	2.50
Group 3											
Bpp-35	5.00	0.60 mM	0.30	0.15	80	2.50	>96.0	>96.0	8.00	1.25	2.50
Bpp-37	10.0	1.25 mM	2.50	0.15	320	5.00	>96.0	>96.0	64.0	5.00	7.50
Bpp-76	10.0	1.25 mM	2.50	0.15	160	2.50	>96.0	>96.0	64.0	10.0	2.50

a) "Notable" refers to a difference of at least 2 twofold dilutions in at least one of the mutants when compared with the parental strain in 2 separate assays (each strain tested in triplicate/assay).

TABLE II.4: Slight[a] Alterations in Antibiotic Sensitivity Of *Bordetella parapertussis* LPS Mutant Strains

BACTERIAL STRAINS	ANTIBIOTIC SENSITIVITY (MIC in µg/ml)							
	Increase in Sensitivity					Decrease in Sensitivity	Mixed Alteration in Sensitivity	
	Chloramphenicol	EDTA	Erythromycin	Gentamycin	Sulphamethoxazole	Polymyxin B	Tetracycline	Ticaricillin
Control Strain								
YRD101	2.50	0.16 mM	0.30	5.00	384	6.20 U/ml	6.25	32.0
Mutant Strains								
Group 1								
Bpp-43	1.25	0.16 mM	0.30	5.00	192	6.20 U/ml	6.25	32.0
Group 2								
Bpp-54	2.50	0.16 mM	0.60	2.50	192	6.20 U/ml	12.5	16.0
Bpp-68	1.25	0.16 mM	0.30	5.00	192	9.40 U/ml	12.5	16.0
Group 3								
Bpp-35	1.25	0.08 mM	0.60	2.50	384	6.20 U/ml	6.25	32.0
Bpp-37	2.50	0.16 mM	0.60	2.50	192	9.40 U/ml	4.70	64.0
Bpp-76	2.50	0.16 mM	0.30	5.00	192	9.40 U/ml	3.12	32.0

a) "Slight " refers to a difference of less than 2 twofold dilutions in at least one of the mutants when compared with the parental strain in 2 separate assays (each strain tested in triplicate/assay).

FIGURE II.1

A) Silver-stained SDS-PAGE profile of lipopolysaccharide from proteinase K-treated Laemmli digests of *B. parapertussis* parental strain, YRD101, and Tn*5*-insert LPS mutants; Group1● - YRD101,Bpp-43. Group2■ - Bpp-54, Bpp-68. Group3▲ - Bpp-35, Bpp-37, Bpp-76.

B) Western immunoblot assay of reactivity of LPS-specific monoclonal antibodies with SDS-PAGE of proteinase K - processed Laemmli digests of *B. parapertussis* parental strain, YRD101, and Tn*5*-insert LPS mutants; Group1● - YRD101,Bpp-43. Group2■ - Bpp-54, Bpp-68. Group3▲ - Bpp-35, Bpp-37, Bpp-76.

  a) Reactivity of *B. parapertussis* O-chain-specific monoclonal antibody A7D12 with SDS-PAGE separated PK-Laemmli digests of *B. parapertussis* standard and mutant strains.

  b) Reactivity of *B. parapertussis* O-chain-specific monoclonal antibody D13B11 with SDS-PAGE separated PK-Laemmli digests of *B. parapertussis* standard and mutant strains.

  c) Reactivity of *B. bronchiseptica* strain 110H+ polyclonal antisera with SDS-PAGE separated PK-Laemmli digests of *B. parapertussis* standard and mutant strains.

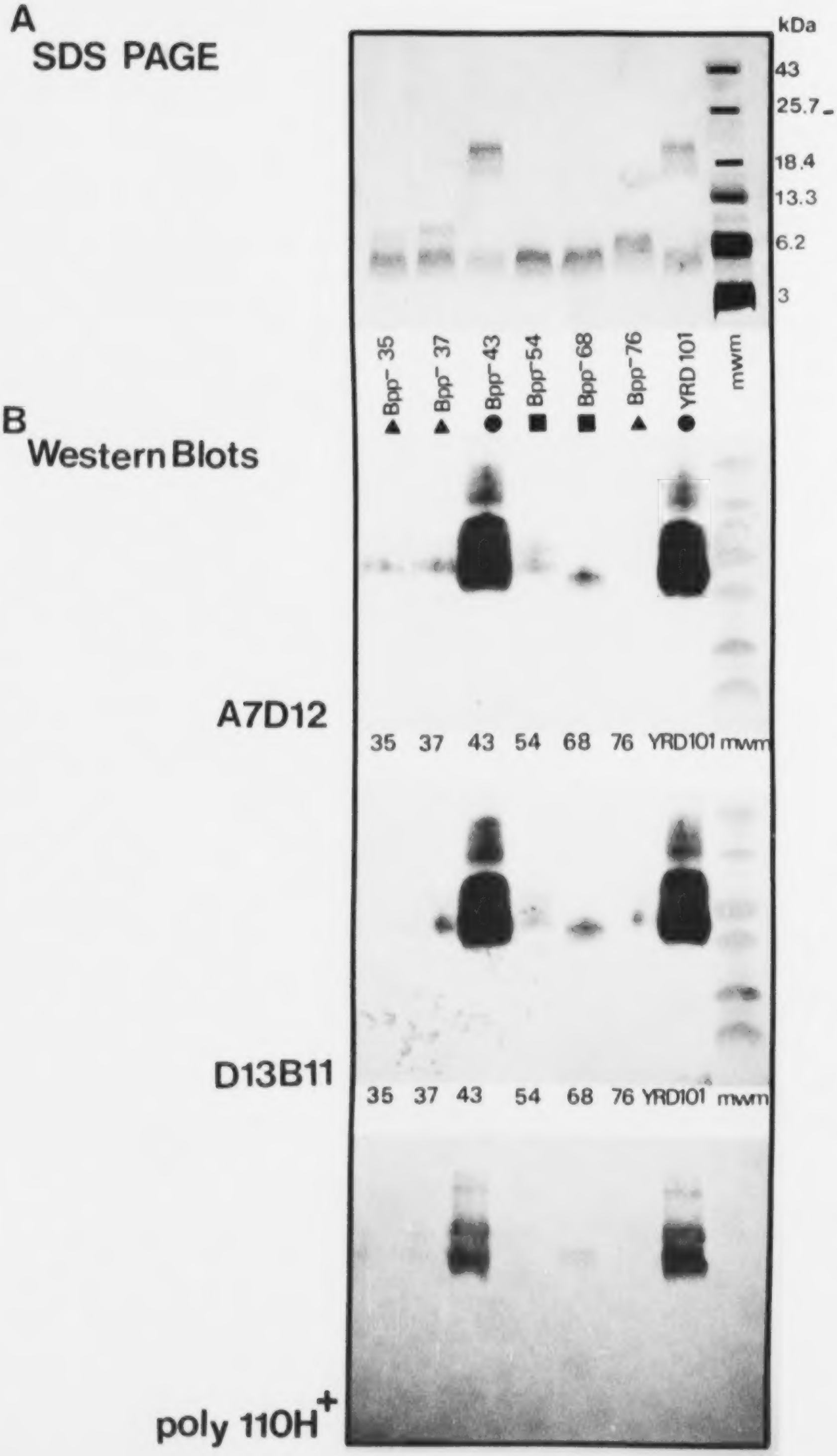

A
SDS PAGE
kDa
43
25.7
18.4
13.3
6.2
3
▲Bpp⁻35
▲Bpp⁻37
●Bpp⁻43
■Bpp⁻54
■Bpp⁻68
▲Bpp⁻76
●YRD 101
mwm
B
Western Blots
A7D12
35 37 43 54 68 76 YRD101 mwm
D13B11
35 37 43 54 68 76 YRD101 mwm
poly 110H+

FIGURE II.2

Southern blot analysis of DNA extracted from of *B. parapertussis* standard and Tn*5*-mutant strains. Autoradiographs of restriction digests probed with a ^{32}P-labeled Tn*5* probe.

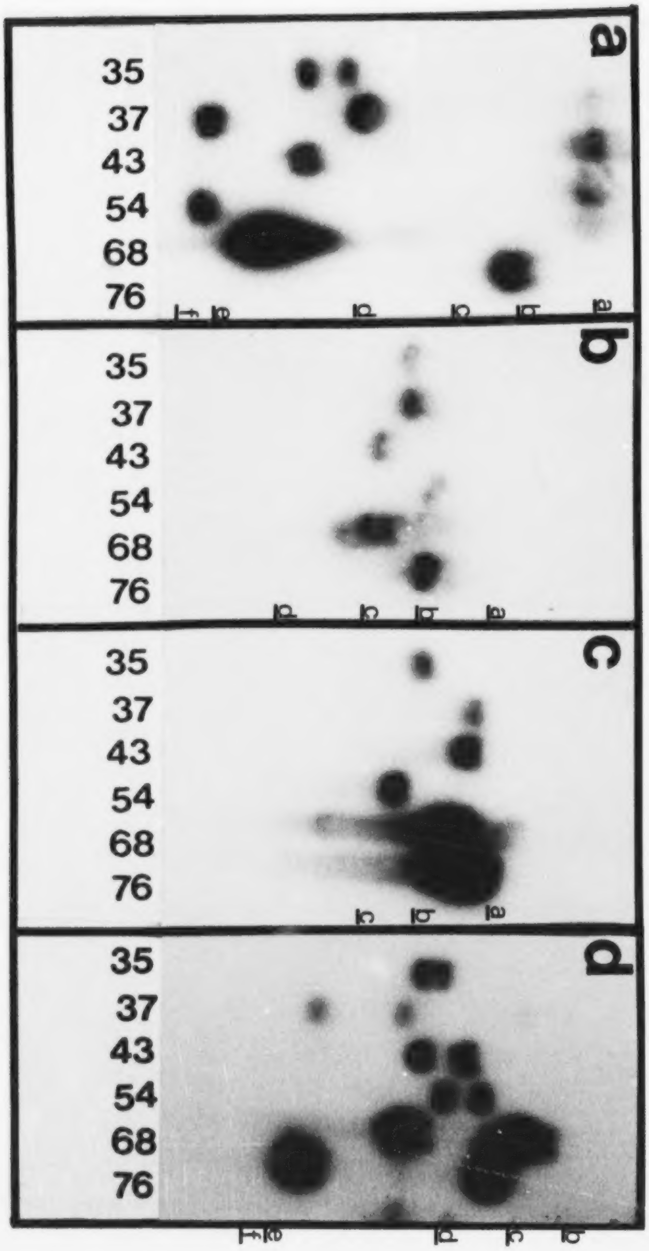
a) DNA restricted with *Bam*H1

b) DNA restricted with *Cla*1

c) DNA restricted with *Eco*R1

d) DNA restricted with *Sal*1

Molecular weight markers are shown as follows (in kb): a) 25.4, b) 9.4, c) 6.6, d) 4.4, e) 2.3, f) 2.0.


a
35
37
43
54
68
76
a
b
c
d
e
f
b
35
37
43
54
68
76
a
b
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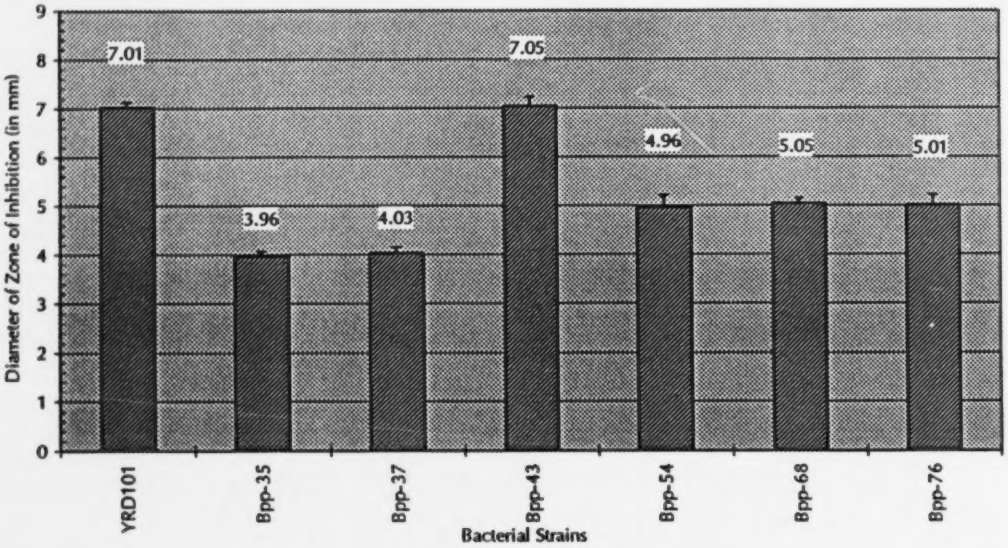
d
c
35
37
43
54
68
76
a
b
c
d
35
37
43
54
68
76
b
c
d
e
f

FIGURE II.3

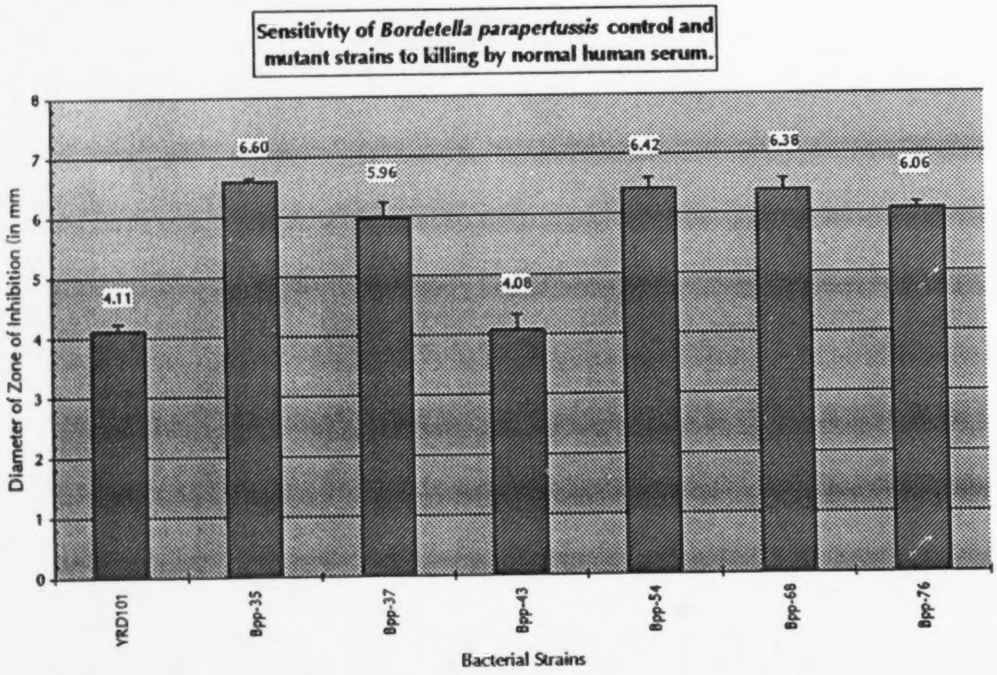
Bactericidal activity of *Bordetella parapertussis* O-chain monoclonal antibody, D13B11, against *B. parapertussis* Tn*5* mutants with altered LPS. Guinea pig serum was diluted 1:4 to a level where bacterial lysis did not occur as the result of incubation with complement, alone. Antibody was added at an ascites dilution of 1:100. Bars indicate standard deviation amongst results of 4 independant experiments done in pentuplicate; that is each sample was tested 5 times for each of 4 independent experiments. $p<.001$ by student t-test for YRD101 vs. all mutant strains save Bpp-43 ($p = 0.7771$).



Bactericidal activity of *Bordetella parapertussis* O-chain mAb D13B11 on *B. parapertussis* mutant strains with altered LPS.

FIGURE II.4

Sensitivity of *Bordetella parapertussis* Tn*5* mutants with altered LPS to pooled normal human sera. Bars indicate standard deviation amongst results of 4 independant experiments done in pentuplicate; that is each sample was tested 5 times for each of 4 independent experiments. p<.001 by student t-test for YRD101 vs. all mutant strains save Bpp-43 (p = 0.8216).



Sensitivity of *Bordetella parapertussis* control and mutant strains to killing by normal human serum.

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CHAPTER III

Construction, Selection And Characterization Of *Bordetella pertussis* Mutants With Tn*5*-Induced Alterations In Lipooligosaccharide.

A. INTRODUCTION

Bordetella pertussis is the causative agent of whooping cough. The cell wall of *B. pertussis,* as in most Gram-negative organisms, is a complex matrix of interacting molecules. The bacteria is distinct from many other pathogens in its utilization of a number of unique toxins and virulence factors, many membrane-associated, in the overall pathogenicity of the disease (45). Characterization of the properties of any one isolated factor yields only limited data due to the collaborative nature of the many molecules involved in *B. pertussis* infection and immunity. Thus, the role of many specific virulence factors have been examined though the creation of genetically mutated *B. pertussis* strains in which a certain toxin or factor has been deleted (15,43). The purpose of the present study was to use Tn*5* insertion in a similar manner to study the effects of alterations of the endotoxin on the integrity of the *B. pertussis* cell wall and thus the cell as a whole.

As a major component of the outer membrane the endotoxin or lipooligosaccharide (LOS) of *B. pertussis* plays a major role in the many interactions which occur between host and pathogen during the processes of infection, disease, and immunity. Neither the direct effects on cell function mediated by the receptor, barrier and molecular exclusion properties of endotoxin, nor the indirect consequences on pathogenicity of LOS interactions with other *B. pertussis* membrane-associated molecules have been determined as complete deletion of the LOS destroys the integrity of the cell wall.

Although the isolated LOS of *B. pertussis* exhibits many of the toxic and immunological properties of the lipopolysaccharide of enteric species (9,17), the lack of commonly associated symptoms such as fever and septicemia in clinical pertussis has resulted in some confusion as to the role of LOS in pathogenicity. Similarly, although implicated in numerous side-effects, the role of LOS has not been definitively established in the reactivity of the current whole cell pertussis vaccine (10,13,17). The properties of *B. pertussis* LOS in its natural environment, interacting with other components of the outer membrane, have yet to be fully explored.

Our intent was to investigate the effect of structural alterations of the LOS on the integrity and function of the *B. pertussis* outer membrane and thus, bacterial pathogenicity. In addition, we hoped to obtain initial data on the genetic regulation of the *B. pertussis* LOS biosynthetic pathway The wild-type LOS of *B. pertussis,* while devoid of the extended O-side chains common in enteric bacteria, does exhibit a limited degree of heterogeneity (9,19,27). Two bands are present on SDS-PAGE, a higher molecular weight component designated LOSA and a minor, lower molecular weight band called LOSB (7,27). Recent findings by Caroff et al have established the source of this heterogeneity as the lack of three terminal amino sugars on the LOSB core (7). These experiments were made possible by the existence of a less frequently isolated clinical strain of *B. pertussis* whose only LOS component is LOSB. We utilized a monoclonal antibody recently developed against LOSB to screen Tn*5* mutants of the wild-type LOSAB strain for LOS alterations (2,23)

Our laboratory has produced ten LOS mutants by Tn*5* insertion, which contain varying quantities of LOSA and LOSB as well as two previously undocumented intermediate LOS types which we have designated IntA and IntB (respective to the band to which they are closer in molecular weight). This study deals with the phenotypic and genetic characterization of these mutants as well as the initial analysis of the effect of these mutations on outer membrane function. A genomic region of approximately 100kb is responsible for the LOS phenotypes completely lacking the LOSA band. The LOS alterations resulted in changes in epitope exposure and thus, the receptor functions of the outer membrane as well as increased sensitivity of the bacterial outer membrane to lysis by human serum. In addition, alterations in phase variation frequency and sensitivity to several antibiotics were demonstrated as the result of the structural variations in LOS.

B. MATERIALS AND METHODS

Strains and monoclonal antibodies

Strains used in this study were *B. pertussis* BP338 and BP347, obtained from A. Weiss (University of Cincinnati, Cincinnati, Ohio, USA), *B. pertussis* MDH134+ was obtained from A. Wardlaw (Dept. of Microbiology, University of Glasgow, Glasgow, United Kingdom) who originally received the strain from the Michigan Department of Health. BP347 is the avirulent Tn*5* mutant which was produced by A. Weiss from BP338, which in turn was selected as a spontaneous nalidixic acid mutant of Tohama 1. Likewise, MDH134- is the avirulent phenotype of MDH134+ which was selected by growth on Stainer Scholte agar. The LOS structure is conserved between virulent and avirulent species of the same strain. The Tn*5*-containing vector pUW964, was constructed and provided by A. Weiss (University of Cincinnati, Cincinnati, Ohio, USA (43)).

Bordetella cultures were maintained on Bordet-Gengou Agar (BGA) with the appropriate antibiotics (Sigma, St. Louis, Mo.) added. *B. pertussis* strain BP338 was maintained on BGA with 60 μg/ml nalidixic acid while Tn*5*-insertion mutants were plated to BGA containing 60 μg/ml nalidixic acid and 25 μg/ml kanamycin. *E. coli* strain HB101 containing the Tn*5* plasmid, pUW964, was maintained on L-agar containing 25 μg/ml kanamycin (33).

LOSA and LOSB-specific mAbs, BL-2 and BL-8, respectively, were provided to us by D. Martin (Unite de recherche en vaccinologie, Croix-Rouge/Recherche et developement, Ste-Foy, Quebec) and were characterized previously (2,23). Ascites fluid containing the mAbs were stored at -20°C, then thawed and diluted in blocking buffer (3% bovine serum albumin in 50mM tris-buffered saline, pH 7.0 (TBS)) for experiments.

B. pertussis growth in broth and LOS extraction.

Initial 100 ml cultures of all *B. pertussis* strains were innoculated from 2 day cultures swabbed from BGA plates. These cultures were grown with aeration for 2 days at 37°C in Stainer-Scholte media with 10 grams/L added casamino acids. The "seed" cultures were then used to innoculate 900 ml of the same media and the broth was isolated for a further 4 days at 37°C with aeration. The LOS was extracted by the standard hot phenol/water method of Westphal and Jann commonly used for *B. pertussis* LOS purification (50) which is detailed in Chapter II. The final pellet was dried, weighed and resuspended to a concentration of 100 µg/ ml. Protein content was analyzed by Coomassie and silver stained SDS-PAGE gels.

Mutation of the wild-type *B. pertussis* genome by random insertion of a Tn5 transposon.

B. pertussis strain BP338 was mutated by random insertion of a Tn*5* transposon. The transposon was transferred though conjugation of the parental BP338 strain with an HB101 *E. coli* strain containing a Tn*5* suicide vector, pUW964 (43). *B. pertussis* strain BP338 was grown for 3 days on BGA containing 60µg/ml nalidixic acid. *E. coli* strain HB101 containing the pUW964 suicide plasmid was grown overnight on L-agar containing 25µg/ml of kanamycin. Each organism was swabbed from its growth plate (normal BGA) onto a mating plate (BGA containing either 10mM $MgCl_2$ or 10mM $MgSO_4$) and incubated at 37°C for 2.5-3.0 h. After incubation the organisms were swabbed from the mating plate to 2.0 ml of Stainer-Scholte broth. Mutants were selected by plating 100µl aliquots of a 10^{-1} dilution of this suspension onto BGA plates containing both 25µg/ml of kanamycin and 60µg/ml of nalidixic acid. This protocol routinely resulted in 100-300 kanamycin-resistant, nalidixic acid-resistant colonies per plate.

Western Colony Blot Analysis

LOS mutants were screened by western colony blot with the LOSB monoclonal antibody BL-8 as detailed in Chapter II. Colonies which reacted with the anti-LOSB mAb, BL-8, were selected from the original plates, subcultured, and their LOS profiles confirmed by SDS-PAGE, silver stain, Western blot and Dot blot analysis.

SDS-PAGE, Silver Stain, Western Blot and Dot Blot Analysis.

Lipooligosaccharide was analysed using *B. pertussis* whole-cell lysates prepared by the method of Laemmli et al.. SDS-PAGE, silver stain and Western blot analysis (with mAbs BL-2 and BL-8) was performed as detailed in Chapter II (16).

Dot blot analysis of the strains was performed using a BioRad Bio-Dot apparatus. One hundred µl of a 0.3 OD suspension (measured at a wavelength of 540 nm (A_{540})) of each strain was applied, per well, to nitrocellulose. The dot blots were incubated with primary then secondary antibodies and developed with NBT/BCIP as described above for western blots.

Antibody Accessibility Radioimmunobinding Assay

The protocol followed was detailed by Martin et al. (2,23). Briefly, 5×10^9 cfu/ml of two day old cultures of *B. pertussis* were incubated with the monoclonal antibody (BL-8 or BL-2) and 0.25µCi of ^{125}I-labeled goat anti-mouse IgG. The quantity of monoclonal antibody bound to each strain of bacteria was measured with a gamma counter.

Preparation of Tn*5* Probes

Probes were prepared by labelling a complete Tn*5* transposon using a random primer insertion method and two different labelling agents (33). A nonradioactive Tn*5* probe was created using digoxigenin as the labelling agent (Boehingher Mannheim). As well, ^{32}P (Amersham) was used to radioactively label Tn*5*. Each probe was similarly reactive for our purposes.

Southern Blot Analysis

Genomic DNA was isolated by the CTAB method (3) as detailed in Chapter II. DNA was restricted for two h at 37°C with five separate restriction enzymes; *Bam*H1, *Cla*1, *Eco*R1, *Sal*1 and *Sma*1. Digests were run on 0.6% agarose gels at 100 volts for 3 h. Southern analysis was carried out as detailed in Maniatis et al. Blots on Hybond-N membrane were probed and developed with the digoxigenin system as described above (33,36) or with ^{32}P labelled Tn*5* probes which were produced using the random primer labelling kit available from Gibco/BRL (Burlington, Ontario).

Pulsed Field Gel Electrophoresis Analysis.

Control organisms, (BP338, BP347) and all ten mutants (MLT 1-10) were analyzed by pulsed-field gel electrophoresis using the restriction enzymes *Xba*I and *Spe*I and following the methods of deMoissac et al. (11). The DNA bands were sized by comparison of migration distances to those of the lambda ladder DNA standard (New England Biolabs, Beverly, Maryland, USA). The position ot the Tn*5* inserts on the *B. pertussis* genome map created by Stibitz et al. was determined by comparison of the *Xba*I and *Spe*I fragments with those of the published PFGE restriction map (37).

To determine which pulsed-field bands contained the Tn*5* insertion, Southern hybridizations were done with a digoxigenin-labeled Dig-Tn*5* probe. The pulsed-field gel was stained with ethidium bromide and photographed followed by depurination in 0.25 M HCl for 15 min to ensure adequate transfer of the DNA to the nylon membrane. The DNA was transferred to Hybond N nylon membrane (Amersham, Oakville, Ontario, Canada) over 24 - 36 h by capillary transfer using the method of Southern et al (33,36) as detailed in Chapter II.

The washed blot was then placed in blocking buffer (digoxigenin blocking reagent (Boehinger Mannheim)) and 10% fetal calf serum in maleate buffer (100mM maleic acid; 150mM NaCl; pH 7.5) for five h at room temperature after which the anti-digoxigenin antibody/alkaline phosphatase conjugate (Boehinger Mannheim) was added at a dilution of 1:10000 for a further incubation of 2-3 h.

A series of six 30 minute washes in maleate buffer preceded the addition of the LumigenPPD detection solution(Boehinger Mannheim). The blot was removed from the LumigenPPD solution, wrapped in plastic wrap, and incubated at 37°C for 15 min It was then exposed to X-OMAT AR autoradiograph film (Kodak, Toronto, Ontario, Canada) for 1-3 h.

The developed film was compared with full size photos of the gel as well as films of the blot probed with the Dig-Tn*5* probe at a less stringent hybridization temperature (37°C). The chomosomal position of the Tn*5* insert was determined from analysis of and comparison with the *Xba*I, *Spe*I pulsed-field maps of Stibitz and Garletts (37).

Bactericidal Assay

Mutants and control organisms were suspended in sterile TGS to an A_{540} of 0.17 - 0.20 and 50 µl of each suspension was transferred to the wells of a 96 well plate. The primary antibody (either BL- 8 or BL- 2) was heated for 30 min at 56°C to destroy any residual complement activity in the ascites fluid. The antibody was then serially diluted ten fold from 1×10^{-1} to 1×10^{-6} and 25 µl of each dilution added, in triplicate, in phosphate buffered saline, pH 7.0 (PBS) to wells of the bacterial suspensions. The organisms were allowed to incubate with the antibody for one hour at 37°C.

Normal guinea pig serum was utilized as a source of complement. The amount of serum causing 50% hemolysis (CH_{50}) was determined using sheep red blood cells sensitized with hemolysin (Cedar Lane) so that a constant amount of complement activity could be used from one experiment to the next. Twenty five µl of a 1: 16 dilution (2 CH50 units) of the guinea pig serum was added to the antibody/bacteria suspension and the mixture was allowed to incubate at 37°C for another hour. Ten microlitres of the total suspension was then put though a series of six 1/5 serial dilutions. Ten µl of each dilution was the plated to BGA and colonies counted after 3 - 4 days.

Complement Fixation

After plating the samples for the bactericidal test, the remaining 90 µl from the bacteria/antibody/complement suspension was incubated with 90 µl of a 3%

solution of sensitized sheep red blood cells (sRBCs) in PBS and incubated for 1 hour at 37°C. The cells were collected by centrifugation for 10 min at 500 x g. and the supernatant was transferred to a flat bottom 96-well ELISA plate. The extent of complement fixation was determined by measuring the amount of hemolysis after incubation of the various bacteria/antibody combinations measured at A_{540}. Results from mutant strains were compared with negative controls which contained no bacteria or no added monoclonal antibody. The positive control used was the combination of *Bordetella parapertussis* 17903 with the *B. parapertussis* specific mAb D13B11 which produced 100% complement fixation and 100% bactericidal killing. The extent of complement fixation was measured as the total complement available (100%) minus the percentage of complement which remained free (after incubation with the mAb and bacteria) to lyse sRBCs.

Radial Diffusion Immunoassay for Assaying Sensitivity to Human Serum.

The method of Fernandez and Weiss was used (personal communication) as detailed in Chapter II. Briefly, fresh, pooled human serum was added to plates containing *B. pertussis*.

Plates were incubated at room temperature for 3 h to allow serum to diffuse. A ten ml overlay of SSA was then added, the agarose was allowed to harden and the plates were incubated for 24 to 48 h at 37°C. Zones of inhibition were measured with the use of Bausch and Lomb stereomicroscope fitted with an ocular micrometer.

Antibiotic Sensitivity Assay

Strains were grown for 2 days on BGA then suspended in 4ml of SSB to an optical density of 0.20 at a wavelength of 540 nm (26). The suspension was then diluted and plated in duplicate to BGA plates to determine the total viable count of organisms used in the innoculum. The agents were selected based on previous studies on the effects of various antibiotics on *Bordetellae* species and enteric endotoxin (4,5,6,15,28, 31,34,39).

Twofold serial dilutions of each antibiotic were performed in duplicate in 96-well tissue culture plates from a stock solution which was predetermined using the parental strain, BP338, as a measure of reference. Control wells with the antibiotic diluent alone, were included in each assay. 100 µl of a 10^{-1} dilution of bacteria was added to the final 100 µl volume of each antibiotic or control (diluent, alone) well. The plates were incubated for 4 days at 37°C. Minimum Inhibitory Concentration (MIC) was measured as the last clear well (no turbidity) both visually and by ELISA reader at an absorbance of 540 nm. Final MIC data was based on geometric mean titres.

Phase Variation Assay

The rate of phase variation was measured by differentiating virulent from avirulent organisms using hemolysis, colony morphology and erythromycin resistance (28,44). Virulent organisms are documented to be domed, hemolytic, and erythromycin sensitive. Avirulent organisms are flat, nonhemolytic, and less erythromycin sensitive. Bacteria were grown at 37°C for two days on BGA then suspended in SSB to an optical density of 0.2 at an absorbance of 540 nm. Dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were made in SSB and 100 µl of each was plated in triplicate to plates of BGA, alone, and BGA with 0.25 µg/ml erythromycin. Plates were incubated for 3 days at 37°C. BGA plates were counted and colonies differentiated on the basis of hemolysis and erythromycin sensitivity. The number of variant colonies (nonhemolytic or erythromycin resistant) were divided by the total colony number on plain BGA to give the frequency of variation.

C. RESULTS

General characteristics of LOS mutants.

All of the LOS mutant strains obtained by Tn*5* insertion demonstrated a colonial morphology which is phenotypically identical to the parent strain, BP338. That is, all mutant strain colonies were domed and hemolytic on BGA. Growth rate, likewise, was unaffected by the presence of the Tn*5* insert. The mutant colonies like the parental strain, BP338, all formed colonies of approximately 1 mm in diameter in approximately three days. As well, the presence of hemolytic activity indicates a retention of parental virulence.

All potential LOS mutants were selected using the BL-8 LOSB-specific monoclonal antibody. BP338 does not react with BL-8 due possibly to a masking of the LOSB by LOSA or the low amount of LOSB present in the BP338 outer membrane. A positive reaction of Tn*5* mutant BP338 colonies (nalidixic acid and kanamycin resistant) with BL-8 indicated that some alteration had occurred in the bacteria which either made the LOSB more accessible or significantly increased the amount of LOSB present. Coomassie and silver stained SDS-PAGE gels of whole cell lysates indicated no alteration in protein content in any of the selected mutants. Of the hundreds of mutant colonies assayed ten mutants were found which reacted positively with the BL-8 mAb.

Analysis of phenotypic alterations in the profiles of the isolated LOS of Tn5 mutants.

Mobility in SDS-PAGE.

Overall, the SDS-PAGE silver stain data indicated a relatively simple pattern of LOS mutation (Figure III.1). Mutant strains MLT3, MLT5, MLT8, and MLT9 possessed abundant LOSA SDS-PAGE profiles similar to that of BP338 while MLT6, MLT7, and MLT10 resembled MDH134+ in more pronounced quantities of LOSB. Mutants MLT1, MLT2, and MLT4 had notable quantities of both LOSA and LOSB and thus, phenotypically resembled both BP338 and MDH134+. Based on mobility of prestained protein molecular weight markers, *B. pertussis* LOS band mobility (LOSA, LOSB, and intermediates) indicated an apparent molecular weight in the range of 4.5 to 5.5 kDa.

In several mutants the LOS profiles were comprised of up to four specific molecular LOS types (Figure III.1). Between the bands commonly accepted as

LOSA and LOSB lay two intermediate species which we have designated IntA and IntB (for intermediate A, the band closest in apparent molecular size or mobility to LOSA and intermediate B, the species closest to LOSB). Where the control LOSs, BP338 and MDH134+, completely lacked these bands, the mutants all contained varying amounts of these intermediate species.

BP338 does possess a well-documented LOSB band which, though minor, was clearly evident on a more heavily loaded, over-developed silver stained SDS-PAGE gel (15,18). These measures unfortunately seriously decreased the clarity of the four individual LOS bands, LOSA, LOSB, IntA and IntB, and this gel is, therefore, not shown on this gel.

Only one mutant, MLT3, was nearly identical to the original parental phenotype, BP338. MLT3, however, had an increase in the concentration of LOSB which can be more easily noted on overdeveloped BL-8 western blots (data not shown). MLT5, MLT8 and MLT9, though possessing a dominant LOSA band, also contained considerable quantities of LOSB and IntA.

MLT1, MLT2,and MLT4 all contained significant amounts of both LOSA and LOSB bands but it was evident that these strains vary in the number and proportion of their LOS molecular species, with MLT2 and MLT4 containing IntA, as well as different concentrations of LOSA and LOSB.

Based on analysis of mobility on SDS-PAGE, the LOS profile of MLT7 was the most comparable to the LOS profile exhibited by the LOSB reference strain MDH134, however the profile indicated the addition of an IntB band as well as the consistent presence of a faint higher molecular weight band of unknown composition. This band has been postulated to be an LOS aggregate or proteinase-K resistant protein contaminant. MLT6 and MLT10 appeared identical to each other on SDS-PAGE. As well, both had IntB bands which are nearly equal in amount to their concentration of LOSB, thus distinguishing them from the standard MDH134 LOSB phenotype. Overall, we obtained nine LOS mutant phenotypes which differ significantly from the only published *B. pertussis* LOS phenotypes, LOSAB (BP338) and LOSB (MDH134).

Western Blot Analysis.

Though all mutant LOS strains were initially selected by reactivity of whole organisms with the BL-8 anti-LOSB monoclonal antibody via colony blot, further examination using three additional immunoassays indicated significant differences between the mutants.

Western immunoblotting qualitatively assessed the relative reactivity of isolated LOS (by Laemmli solubilization and proteinase K digestion) of the various mutant and standard strains with monoclonal antibodies specific for LOSA (Figure III.2(a))and LOSB(Figure III.2(b)). In consideration of the subjective nature of this assay several western blots were run on the mutants with minor variations in the standard assay conditions, most notably development time.

The data tabulated in Table III.1 represents data from an average of five western blots which were varied slightly in development time, blocking buffer, antibody dilution, etc. as a means of determining the most accurate account of each mutants' reactivity with the LOSA and LOSB monoclonal antibodies. As such, there are minor differences between the overall, averaged data contained in this table and figures IV.2(a) and IV.2(b), notably the decreased reactivity of MLT4 and MLT5 to BL-8 as presented in Table III.1. The averaged blot results serve as a point of comparison for the reactivity of the isolated LOS of the various mutants in Western blot analysis with the reactivity of the LOS as presented on intact bacteria (dot immunoassay, accessibility data and complement fixation).

Generally, western blot results correlated well with SDS-PAGE profiles. If there were large amounts of LOSA or IntA present in the SDS-PAGE profile then the mutant reacted strongly with BL-2. If large LOSB or IntB bands were evident then the isolated LOS reacted more strongly with BL-8. For example, the MLT4 reacted less strongly than MLT2 with BL-8. This concurred with SDS-PAGE analysis where more LOSA could be seen in MLT4 than in MLT2 (Table III.1, Figure III.1).

Similarly, mutants MLT3, 4, 8, and 9 bound more strongly with the LOSA mAb BL-2, as could be expected from the large LOSA and IntA bands which were present in their SDS-PAGE profiles. Mutants whose SDS-PAGE patterns showed major LOSB and IntB bands such as MLT6, 7, and 10 were more reactive with the LOSB mAb, BL-8. The presence of both LOSA and LOSB in large amounts, as was indicated by the SDS-PAGE profile of MLT1 was confirmed by its strong reaction to both monoclonal antibodies.

Overall, the isolated LOS of the various mutants reacted to the LOSA and LOSB mAbs as would be expected from analysis of their individual SDS-PAGE LOS profiles.

Growth in Culture and LOS Extraction of Control and LOS Mutant Strains.

All strains grew at similar rates in broth culture. Upon standard extraction with hot phenol/water, however, there was a distinct drop in LOS yield for those strains which had large amounts of LOSB (Table III.2). As is the case for MDH134+ (approximately 39%), the virulent clinical isolate whose lipooligosaccharide consists mainly of LOSB, strains such as MLT7 and MLT10 extracted poorly with the hot phenol water protocol commonly used for *B. pertussis*, approximately 14% and 19% of BP338 gm yield, respectively. Mutant strains which possessed LOSA in large quantities extracted as well as the parental phenotype, BP338, with comparative yields of approximately 76% (MLT4), 81% (MLT2, MLT5), 86% (BP347), and 109% (MLT1).

Analysis of alteration in LOS as presented in the intact cell.

Dot Blot Analysis and Radioimmunoassay Analysis

The dot immunoassay (Table III.1) and the ^{125}I radioimmunobinding assay (Figure III.3, Table III.1) measured the ability of the LOSA and LOSB-specific monoclonal antibodies to bind to the LOS as presented in intact organisms. The two assays showed agreement within each strain and were, for the most part, consistent with the SDS-PAGE and Western blot profiles. For example MLT1 had almost equal amounts of LOSA and LOSB on SDS-PAGE and reacted equally well with both BL-8 and BL-2 in the whole cell assays.

Mutants MLT2, MLT4 and MLT5, however, were the exception in that these mutants showed some variation in their overall reactivity to the LOSA and LOSB monoclonals in the whole cell assays. Isolated MLT2 LOS reacted better with BL-8 where intact MLT2 reacted better with BL-2. MLT4 and MLT5 reacted strongly with BL-8 in the ^{125}I-mAb radioimmunoassay only.

As well, MLT2 and MLT5 reacted far less strongly with the LOSA monoclonal, BL-2, in the western blot than they did in the other whole cell immunoassays. The isolated LOS in the western blot analysis of these two mutants likewise appeared to react differently to the LOSB specific mAb, BL-8, in this case better, than would have been expected from the whole-cell immunoassays. These discrepancies are possibly due to the presence of intermediate LOS species (IntA and IntB) and the difference in species proportion as indicated in the SDS-PAGE profile. Another explanation could be a mutation-related change in surface exposure of the BL-2 and BL-8 epitopes.

Other than these discrepancies, results from the two immunoassays correlated well with data from SDS-PAGE analysis. For this reason, Table III.1 is organized on the basis of SDS-PAGE LOS type (column 1) for reference and comparison.

Genetic analysis of Tn*5* location in genomic DNA of LOS mutant strains.

Southern Blot Analysis

The presence of a single Tn*5* insert in each of the mutant strains was confirmed by Southern analysis (Figure III.4). The Tn*5* probe hybridized with a single band in mutant and control strains when DNA was digested with enzymes such as *Cla*1 and *Eco*R1 which do not recognize sites within the Tn*5* gene. When digested with enzymes such as *Bam*H1, *Sma*1 and *Sal*1 which have a single cut site within the Tn*5* gene each DNA digest produced two bands which hybridized with the Tn*5* probe. The lack of two distinct bands with digests of BP347 is likely due to a similarity in size of the two restriction fragments and the poor resolution of small differences in such large fragments when using a 0.6% agarose gel system.

Genetic homology was indicated in only two instances. Restriction digests of MLT5 and MLT6 with all five enzymes yielded Tn*5* hybridized bands of similar sizes. This was also the case for digests of MLT7 and MLT8. This similarity in band size for five different restriction enzymes indicates a strong similarity between the structure of the genes in which the Tn*5* is inserted in MLT5 to that in MLT6 and the Tn*5* insert gene in MLT7 to that in MLT8. However, the pulsed field data indicates that the Tn*5* insert gene in MLT5 is not close to that of MLT6 nor are the Tn*5* genes located in a similar area for strains MLT7 and MLT8. To ensure that the strains were not somehow switched simultaneous preparations of each strains' LOS and genomic DNA (in agarose blocks for pulsed field gel analysis and free in solution for southern analysis) were prepared from the same bacterial suspension. SDS-PAGE, Southern and pulsed field gel analysis were performed concurrently for each strain and the results of the initial analysis were confirmed.

Pulsed Field Gel Analysis

Agarose blocks of the genomic DNA of mutant strains were restricted with *Xba*I and *Spe*I then analyzed by pulsed-field gel electrophoresis. Incubation of the blotted pulsed-field gels with a digoxigenin-labelled Tn*5* probe yielded the approximate genomic location of each Tn*5* insertion (Figure III.5).

As all mutants were derived from BP338, we were able to use the pulsed-field gel map created by Stibitz et al. (37) to locate the disrupted genes on the *B. pertussis* genome using a Tn*5* probe (Table III.3). By coordinating the band location of the Tn*5* in *Xba*I digests of the mutants with the Tn*5* location in a *Spe*I digest of the mutants, the general location of the genes responsible for the LOS phenotypic changes was determined. Overall, the Tn*5* was located in 5 different regions for the ten mutants based on *XbaI/Spe*I fragment positions.

Tn*5* insertions in strains MLT2, MLT6, MLT7, MLT9, and MLT10 all mapped to an approximately 100 kb region at approximately 3200-3300 kb on a 3750 kb map of the *B. pertussis* chomosome (*Xba*I fragment A/*Spe*I fragment J2). MLT1 and MLT4 map to the area immediately upstream of this region at approximately 3000-3200kb in a 200 kb region (*Xba*I fragment A/*Spe*I fragment C). MLT3 is located in another approximately 100 kb region between 2900 - 3000 kb along the map

(*Xba*I fragment L4/ *Spe*I fragment C). The Tn*5* insert in MLT8 maps to a 80kb region slightly further from the main LOS gene cluster at approximately 2750-2820kb (*Xba*I fragment H/*Spe*I fragment E3). MLT5 maps to a 240 kb genomic region located at approximately 60-300 kb from the origin (*Xba*I fragment E/*Spe*I fragment E1).

Analysis of Alterations on Bacterial Outer Membrane Function.

Alterations in Complement Fixation and Bactericidal Activity of BL-2 and BL-8 mAbs in the Presence of Guinea Pig Complement.

The basic pattern of reactivity in the complement fixation assay was consistent with data from the radioimmunobinding accessibility data (Figure III.6). For example MLT1 again reacted with both mAbs indicating the strong presence of both LOSA and LOSB in its structure. MLT3 fixed slightly less complement with the LOSB mAb, BL-8, than would have been expected from the accessibility data. The only major difference noted was the lower amount of complement fixed by MLT2 in the presence of BL-2 and BL-8 than would have been expected from the accessibility data. This is not inconsistent, however, as Western blot analysis also indicated a dual mAb reactivity for MLT2 (reactivity with both LOSA and LOSB).

None of the mutant (MLT1 to MLT10) or control strains (BP338, BP347, MDH134+, MDH134-) were susceptible to the bactericidal activity of complement in the presence of either mAb (data not shown). Thus, the complex formed by the mAbs with complement was not capable of initiating bacterial lysis under the conditions used in this study.

Alterations in Sensitivity of LOS Mutants to Pooled Human Serum.

As previously documented the sensitivity of the virulent *B. pertussis* strain BP338 was consistantly lower than for the avirulent strain BP347. MDH134+ was found to be less sensitive than BP338. The sensitivity of the mutants, however, varied significantly with the origin of the serum being used in the assay. Several different sources or serum were utilized for these assays and although the difference between the control strains, BP338, and BP347, remained consistent the differences between the mutants, fluctuated depending on the source of the serum. Data from different individuals resulted in different rankings of the mutants for sensitivity. The mutants were consistantly different from the control strains, however, the degree to which each mutant differed tended to vary from one individuals serum to the next. Results with the largest pool of serum (sera was taken from 8 different individuals, then pooled) are documented in Figure III.7.

The general trend established three subgroups of sensitivity within the mutant population. MLT3 and MLT9 were the least sensitive of the mutants, less than BP338 but more so than MDH134+ which was the most resistant of all strains tested to killing by human serum. MLT2, MLT4, and MLT5 were comparable to the parental strain, BP338, in sensitivity to human serum. MLT1, MLT6, MLT7, MLT8, and MLT10 were more sensitive to human sera than BP338 but less so than BP347.

Alterations in Frequency of Phase Variation of LOS Mutants.

B. pertussis is capable of switching from virulent to avirulent phenotype under two distinct set of circumstances. Phase variation is the term given to the process by which approximately 1 in every 10^6 cells shifts to avirulence due to the occurance of a single characterized frameshift mutation. Antigenic modulation refers to the phenomenon in which a phenotypic shift of the entire culture occurs due to environmental stimuli.

During the course of these investigations significant differences were noted in the rate of phase variation of several of the LOS mutants. Standard passaging of cultures revealed a predisposition for loss of hemolytic activity in several of the mutants. The nonhemolytic colonies did not revert to the hemolytic phenotype readily thus the phenomenon was attributed to phase variation as opposed to antigenic modulation. When the actual rate of phase variation was measured it was found that MLT2, MLT3, and MLT5 all varied at higher rates than the parental strain. The phase variation frequency rates of these mutants were 2.6×10^{-4}, 3.4×10^{-4}, and 1.5×10^{-2}, respectively. The frequency of variation for BP338 was 2.9×10^{-6}.

To confirm that the loss of hemolysis was indicative of a phase shift from virulence to avirulence as opposed to a disruption of the hemolytic activity, alone, a second phase variation assay utilizing the heightened sensitivity of virulent organisms to erythromycin was used.

MLT5 produced no more erythromycin resistant colonies than did the virulent parental strain, BP338. The frequency of colonies which grew on erythromycin as opposed to BGA, alone, were 3.1×10^{-6} and 4.3×10^{-6}, respectively. MLT2, and MLT3, however, had frequencies of 1.7×10^{-3} and 1.5×10^{-4}, respectively. The increases in erythromycin resistant phase variants were consistent with the increased number of nonhemolytic organisms although not equivalent to the frequency of BP347 organisms which grew on erythromycin; 3.9×10^{-1}.

Alterations in Sensitivity of LOS Mutants to Antibiotics and Outer Membrane-Active Agents.

The sensitivity of the LOS mutants to numerous antibiotics and membrane-active reagents was assayed and compared to results obtained with the virulent parental strain BP338, its avirulent counterpart BP347, and the virulent LOSB strain MDH134+ (Table III.3). Five strains were chosen from the original ten to provide a diverse range of LOS alterations; MLT1 (which contained equal amounts of LOSA and LOSB), MLT2 and MLT5 (which contained varied amounts of LOSA, IntA and LOSB), MLT10 (which contained only IntA and LOSB), and MLT7 (which contained only IntB and LOSB). In all instances total bacterial viability counts were between 3.0×10^{7} and 2.0×10^{8} cfu/ml. A difference in antibiotic sensitivity of 2 dilutions or more was considered a notable alteration in sensitivity (Table III.4) while smaller differences were considered slight alterations in sensitivity (Table III.5).

BP347 was significantly less sensitive to several antibiotics than was the virulent strain, BP338. Notable decreased sensitivity to amikacin and kanamycin was expected due to the kanamycin-resistance gene encoded by the Tn*5* insert. The decreased sensitivity to streptomycin is not as clearly attributable to the kanamycin resistance of the transposon but has been documented as an expected result of Tn*5* insertion in previous studies (43). BP347 also exhibited notable decreases in sensitivity to oxacillin, cefotaxime, cephalothin, erythromycin, fusidic acid, oleic acid, rifampicin, tetracycline, and vancomycin and slight decreases in sensitivity to ampicillin, and trimethoprim. In comparison with BP338, BP347 exhibited notable increases in sensitivity to sulphamethoxazole and novobiocin. No notable difference was observed in the sensitivities of the control strains to the LPS-specific agents, EDTA, EGTA, and polymyxin B. MDH134+ displayed identical sensitivity in comparison to BP338 with erythomcin, novobiocin, oleic acid, tetracycline and EDTA. Slight increases in sensitivity were noted in assays testing fusidic acid, rifampicin, amikacin, kanamycin, streptomycin, ampicillin, cephalothin, vancomycin and EGTA. Notable increases in sensitivity were noted for sulphamethoxazole, trimethoprim, and cefotaxime.

Only a few notable discrepancies in antibiotic sensitivity were conspicuous in comparing the LOS mutants to the parental strain, BP338. However, numerous slight yet consistent differences were documented. All of the mutants demonstrated slight decreases in their sensitivity to ampicillin, and oleic acid as well as slight increases in sensitivity to cefotaxime, EDTA, and EGTA.

Most of the LOS mutant strains demonstrated slightly decreased sensitivity to the hydrophobic antibiotics; erythromycin, fusidic acid, and rifampicin as well as notably decreased sensitivity to the hydrophilic antibiotics; sulphamethoxazole and trimethoprim. Notable increased sensitivity to novobiocin was documented as well as a slight increase in sensitivity to the large molecular weight peptide antibiotic; vancomycin and the hydrophillic antibiotic; cephalothin.

MLT1 was a conspicuous exception in its consistent similarity to the parental strain, BP338. There were no obvious differences in the sensitivities of MLT1 to erythromycin, fusidic acid, rifampicin, sulphamethoxazole, trimethoprim, or vancomycin.

As well, MLT2 showed no difference from BP338 in sensitivity to fusidic acid or cephalothin and MLT10 was similar to BP338 in its reaction to novobiocin.

D. DISCUSSION

Isolated *B. pertussis* lipooligosaccharide (LOS), like the lipopolysaccharide (LPS) or endotoxin of gram-negative enteric bacteria, exhibits toxicity in vivo. By these properties, it is thought to be associated with the symptoms of fever and discomfort often associated with administration of the whole cell vaccine (9,10,13). Little has been definitively determined, however, as to the exact nature of its role in either vaccine reactivity (systemic and local effects) or *B. pertussis* pathogenicity. This may be due, in part, to the structural and functional differences between the isolated LOS molecule and LOS as it is presented in the bacterial outer membrane. The Gram negative outer membrane forms the interactive surface by which host/pathogen correspondence occurs. As endotoxin is a major and integral component of this matrix the structure and possibly, the function, of the toxin is affected by numerous associations with other outer membrane molecules. Thus, the conformation and structure of the cell-presented LOS differs significantly from that of isolated LOS. Endotoxin presents in three general conformations in the outer membrane; in a lamellar orientation, as hemi-micelles complexed with proteins and as hemi-micelles introduced by divalent cations and/or polyamines (18,42). It is therefore important that investigations examine the native molecule as presented with its native associations rather than study the properties of the isolated toxin, exclusively, if a true concept of the role of endotoxin in pathogenicity is to be evolved.

The goal of this study was to create and study a number of *B. pertussis* strains with genetically altered LOS which differed from the naturally existing LOSAB and LOSB phenotypes. By characterizing and analysing the properties of these specifically altered bacteria we hoped to clarify the role of LOS in outer membrane mechanics and thus, *B. pertussis* infection and immunity. It is possible to modify the LPS in an effort to examine its effect on neighbouring molecules and the cell as a whole as was done in the creation of the "rough" LPS chemotype mutants of *Salmonella typhimurium* (21,24). Mutations resulting in progressively truncated LPS molecules produced significant changes in the pathogenicity of the organisms, notably; increased sensitivity to complement-mediated killing as well as various antibiotics. The recent availability of a monoclonal antibody (mAb) specific for the mutant LOSB phenotype allowed for an efficient, specific selection of LOSB mutants which had been genetically "tagged" with a Tn*5* transposon (23).

This method of mutation and screening has produced ten Tn*5* mutant strains derived from the wild-type LOSAB strain BP338 which react with the LOSB mAb, BL8, to a much higher degree than the parental phenotype. Characterization of these mutants indicated the existence of four (LOSA, IntA, IntB, LOSB) rather than the two previously reported LOS bands (LOSA and LOSB) distributed over nine different LOS phenotypes (of the ten mutant strains tested, MLT6 and MLT10 appeared to be very similar in phenotypic and genetic characteristics and could possibly be the result of an insertion in the same gene).

The LOS mutants developed in our laboratory showed SDS-PAGE structural profiles which were consistent with their antigenic reactivity (alteration in epitope exposure). The LOSA, IntA-dominant phenotypes reacted well with BL-2 and more poorly with BL-8 while the LOSB, IntB-dominant mutants reacted better with BL-8 than BL-2 (Table III.1). There were only a few anomalies. MLT3, though almost identical to the parental strain BP338, was picked up by initial colony blots with BL-8 due to the increase in LOSB concentration which is clear on heavily developed western blots and SDS-PAGE.

The only mutant to exhibit an overall inconsistency in its antigenic reactivity was MLT2. This mutant fixed a lower amount of complement in the presence of BL-2 than would have been anticipated from accessibility data. As well, there was a noted difference in reactivity of the LOS monoclonals with isolated LOS in comparison with the membrane-associated LOS found in intact bacterial cells.

Some of these differences may be the result of differences between the presentation of isolated LOS separated then blotted from a gel and the presentation of LOS associated with the whole organism as is the case with the dot blot and accessibility data. IntA could be more reactive with the LOSA-specific BL-2 when presented in the whole cell, while simultaneously masking the LOSB epitope which reacts with BL-8. Upon disruption the LOSB would be more exposed and the change in presentation or availability might make the IntA less similar to LOSA, decreasing the reactivity of the BL-2. Differences in the IntA and LOSA proportions could explain the differences in reactivities among IntA containing mutants.

This hypothesis rests on the IntA and IntB being reactive with the BL-2 and BL-8 mAbs. Attempts to transfer the separated IntA and IntB have not maintained the band resolution required to definitively prove the exclusive binding of these new LOS forms with BL-2 and BL-8. Blots with BL-8 followed by a second reaction with polyclonal BP338 antisera, however, have distinguished between the upper LOSA/IntA polyclonal BP338-reactive bands and the lower IntB/LOSB mass which reacts to BL-8 (data not shown).

Analysis of the LOS mutants indicate that a major region responsible for genetic coordination of much of the observed phenotypic LOS heterogeneity is the area which stretches from approximately 2900 kb to 3200 kb on the 3750 kb genomic map, and encompasses genes responsible for MLT1, MLT2, MLT3, MLT4, MLT6, MLT7, MLT9, and MLT10. This region also contains the structural gene responsible for pertussis toxin production (37). The consequences of this genetic association, if any, are unknown.

Pulsed field mapping results of several Tn*5* tagged LOS mutants indicated a clustering of the putative LOS genes which was found to mirror phenotypic similarities. Four of the ten mutants, MLT2, MLT6, MLT7, and MLT10 which contained large amounts of LOSB or IntB and little, if any LOSA, were found to have genetic origins in a chomosomal region between 3100 - 3200 kb. A second group comprised of strains such as MLT1 and MLT4 which contained large amounts of both LOSA and LOSB had Tn*5* inserts in the same genomic region while those mutants whose LOS were comprised mainly of LOSA, (MLT3, MLT5 MLT8 and MLT9) had Tn*5* inserts which were less localized. Isolation and sequencing of the genes containing the transposon inserts will be the first documentation of the previously unexamined genetic organization of *B. pertussis* lipooligosaccharide biosynthesis.

Southern analysis indicated a substantial amount of structural homology between the genes in which the Tn*5* was inserted for two pairs of mutants; MLT5 and MLT6, and MLT7 and MLT8, respectively. Pulsed field mapping did not place these mutants in a similar genomic location, however, strengthening the phenotypic evidence that each of the mutants in the pairs were the result of different gene disruptions.

Molecules such as endotoxin which is comprised of carbohydrates and lipids have nonlinear genetic relationships, ie. DNA does not directly encode the molecule. A host of proteins intermediates are responsible for assembly and transport of the toxin. In *Salmonella typhimurium* the biosynthesis of LPS has been demonstrated to be organized into discrete "blocks" of genes (as termed by Makela and Stocker, (22)) which coincide with the required function of a corresponding "block" of gene products. For example the genes required to synthesize the O unit are found in one cluster on the genome while another cluster responsible for core construction is found elsewhere. Separate blocks are believed to exist for construction of the primary components of the LPS (the O unit, core and lipid A), the final assembly of the entire molecule, the addition of "accessories" to the finished molecule and the regulation of the entire process. Additionally, LPS biosynthesis involves numerous "housekeeping" genes for common precursor sugars, phosphylation, etc. which are scattered around the genome.

As 80% of all the mutants created result from a disruption in the 300kb area between 2900kb and 3200kb on the 3750 kb genome we postulate that the block of genes responsible for the assembly of the three sugar "O-antigen" of *B. pertussis* resides in this region, possibly as an operon. Whether the three sugars can indeed be termed an O unit and form a distinct block which is separate from that of the core sugars has yet to be determined. Thus, the assembly of the core may also be governed by this gene "block".

It is probable that the mutants are the result of a disruption in genes encoding the construction or regulation of either N-acetylglucosamine, 2,3-di-N-acetyl-2,3-dideoxyhexuronic acid, or N-methyl-N-acetylfucosamine (Figure I.2). These are the sugars which comprise the proposed O antigen monomer which is found in LOSA but is lacking in LOSB (17,18). From our initial phenotypic and genetic characterization of the mutant *B. pertussis* strains we speculate the possible identities of IntA as a LOSA molecule lacking the N-acetylglucosamine and of IntB as a LOSA lacking both N-acetyl glucosamine and 2,3-di-N-acetyl-2,3-dideoxyhexuronic acid. The total loss of a sugar could occur by a Tn*5*-mediated disruption of either the glycosyltransferase responsible for transfer of the sugar to the growing chain or of an enzyme required in biosynthesis of the sugar.

As well, the variation in ratio of LOSA to LOSB (which is most notable in MLT1) presents the possibility of a regulatory as well as a structural role for some of the Tn*5*-altered genes. These proportional differences could be the result of a "leaky" mutation or due to the alteration or deletion of any of a number of enzymes in or associated with the LOS biosynthetic pathway. The Tn*5* insert may also be causing multiple polar effects if it inserted upstream of an operon (ie. in a promoter, etc.). The cloning, sequencing and expression of the Tn*5* tagged genes should help clarify the exact nature of the gene disruption responsible for the observed phenotypic changes.

We were suprised that we failed to obtain any pure LOSB exclusive phenotypes as have been reported by other laboratories (i.e. MDH134, A100) (7,23). Why intermediate LOSs IntA and IntB, which have never to our knowledge been reported outside this laboratory, were produced while the more clinically established, spontaneous LOSB phenotype was not, is at this point, a matter of conjecture. Perhaps the exclusive LOSB phenotype is the result of the manipulation of more than one mutation in the LOS biosynthetic pathway. Further evaluation and possibly further mutation of the mutants we have created will shed some light on this issue.

Several studies have characterized the ability of Gram negative endotoxin to activate complement and form the membrane attack complex which in turn leads to cell lysis (2,4,9,20). Thus, assays examining the ability of the variant strains to fix complement in the presence of the LOSA and LOSB mAbs served as initial attempts to characterize the consequences of LOS alterations on an important function of the outer membrane. Unlike physical assays such as SDS-PAGE analysis and Western blotting, the variation in complement fixation by the mutant LOS strains from the parental strain provided the first rudimentary correlation of structural LOS alteration with changes in bacteria/host interaction. In organisms which have extensive O units the loss of the most exposed sugars often leaves the cell vulnerable to complement-mediated killing. The effect on *B. pertussis,* which has a very truncated O-chain, was different. Although the mutants were able to fix complement with the appropriate (ie. reactive) monoclonal antibodies the complex formed was unable to initiate membrane lysis under the experimental conditions utilized.

As this was possibly due to the nature of the monoclonal antibodies it was decided that an assay of more clinical relevance might be of interest. The sensitivity of the mutants to whole human serum was assayed. Recent studies by Fernandez and Weiss characterized a virulence-regulated locus, *brk* (Bordetella resistance to killing), the products of which, BrkA and BrkB, cause an increase of at least 10 fold in the resistance of *Bordetella pertussis* to classical pathway-mediated complement killing by human sera (12). BrkB is thought to be situated on the cytoplasmic membrane while BrkA is an outer membrane protein. Most LOS mutant strains differed from both BP338 and BP347. The alterations in a specific mutant may be the result of a disruption of *brk*-regulated resistance (ie. conformational alteration of BrkA) or the effect of an unrelated system. Interestingly, although the strains with altered LOS were consistantly different from BP338 and BP347 in sensitivity to complement-mediated killing by human sera the degree of this sensitivity varied from one individual's serum to another for each mutant strain.

If *brk*-regulated resistance plays a role in the alterations in sensitivity observed in the LOS mutants this variation amongst individuals could be attributable to the antibody-dependence of the classical complement pathway. Antibodies to the appropriate *B. pertussis* epitopes must be present to properly form the membrane attack complex with complement and fully activate the pathway cascade. By modifying a major outer membrane constituent such as the LOS it is probable that the required epitopes have been modified as well. Different individuals would, through different levels of exposure to wildtype *B. pertussis,* possess different arrays of antibodies which are reactive to *B. pertussis* epitopes. The combination of variations in epitopes required on the mutant membranes and antibodies available in the serum likely contributed to the heterogeneity witnessed in the responses of individual sera to different mutants. The consistent contrast of individual responses to BP347 and BP338 in comparison with the LOS mutant strains suggests an important role for LOS in the sensitivity of *B. pertussis* to killing by human serum and thus, a heretofore unexplored role in pathogenicity and infection.

The effect of the LOS alterations on bacterial defense systems and membrane barrier functions was further explored by analyzing the effect of numerous antibiotics and cell membrane reactive agents on the mutants in comparison with control and parental strains. As the first erythromycin-resistant *B. pertussis* strain has recently been isolated the mechanism of interaction between various antibiotics and the outer membrane is of significant relevance (18,25). The most drastic differences documented in studies of the *Salmonella typhimurium* Ra-Re LPS mutants are the 90-98% increase in sensitivity of the mutants to the hydrophobic antibiotic, novobiocin and the 100-500% decrease in sensitivity to the hydrophilic antibiotic tetracycline (31,34). All of the *B. pertussis* LOS mutant strains but MLT10 exhibited comparable alterations in novobiocin and tetracycline sensitivity (81-88% increase in sensitivity and a 300-400% decrease in sensitivity, respectively) as well as alterations in a number of previously unexamined antibiotics. Organisms such as *S. typhimurium* are thought to become more sensitive to hydrophobic antibiotics as the result of the creation of "patches" of phospholipid bilayer in an effort to structurally compensate for the loss of endotoxin-associated protein. A decrease in susceptibility to hydrophilic antibiotics is also linked to the loss of such proteins: porin transport proteins. Endotoxin-associated proteins are lost when an alteration in LPS structure destabilizes the noncovalent bonding of endotoxin to an adjacent molecule. This process occurs at precise structural points in both *Salmonella* (loss of terminal glucose from Rc mutant to produce Rd_1 mutant) and *E. coli* (loss of phosphate groups on heptose in D21e7 mutant) (25,42). It must be noted that the relative structural truncation was far less drastic in the *B. pertussis* LOS mutants than the enteric species (34,37). As well, the chemical structure of *B. pertussis* LOS differs substantially from those endotoxins with naturally occurring extensive O-side chains. The LOS of *B. pertussis,* naturally bereft of an extensive O unit is electrostatically reinforced with divalent cations and a high degree of negative charges on its polysaccharide portion. The loss of even a small number of these charges could result in a noticeable change in the interactions of LOS with LOS as well as other molecules.

Overall, LOS mutant strains exhibited notable alterations in sensitivity to both hydrophobic and hydrophilic antibiotics (Table III.4). All mutant strains except MLT10 were notably more sensitive to the hydrophobic antibiotic novobiocin as well as a decrease in sensitivity to the hydrophobic agent, oleic acid. *B. pertussis* is distinct from many other Gram-negative organisms in its increased sensitivity to fatty acids such as oleic acid. This increased sensitivity is not fully understood but the data here suggests a role for the LOS in the bacterial sensitivity to fatty acids which so complicates the in vitro culture and isolation of *B. pertussis*. In addition to tetracycline, notable decreases in sensitivity were also detected to the hydrophilic antibiotic sulphamethoxazole. As previously mentioned, hydrophilic molecules pass though the Gram-negative outer membrane by way of protein porins. It is possible that the alterations in LOS structure have disrupted the conformation of associated porin structures, limiting their ability to transport certain size molecules, as has been the case in several other studies (25,34,37,42). The consistent differences of the LOS mutants from the parental strain in sensitivity to antibiotics of varied chemical properties indicates a disruption in overall membrane integrity which was enough to allow alterations in the passage of hydrophilic as well as hydrophobic agents into the cell. Thus, changes in the membrane are the result of more than alterations in the gross structural barrier properties of the LOS polysaccharide moiety, alone.

Phase variation is the phenomenon by which a shift from virulent to avirulent phenotype occurs due to a specific frameshift mutation. It involves a number of outer membrane-associated receptors, factors and toxins. *B. pertussis* LOS, however, has never been associated with this process. As an unexpected result of the Tn5 mutation a number of LOS mutants were observed to undergo this phenomenon at a higher frequency than the parental strain. Two distinct characterisitics of the phase variation shift from virulence to avirulence were used to confirm these increases; the lack of hemolysis which occurs upon shifting to an avirulent phenotype and the decrease in sensitivity to erythromycin. Hemolysis is controlled by *adc,* the adenylate cyclase toxin - hemolysin gene, which in turn is controlled by a trans-acting *vir* operon during phase variation. Three mutant strains demonstrated an elevated frequency of shifts from hemolytic (virulent) to nonhemolytic (avirulent) phenotypes. To differentiate between disruptions in the phase variation phenomenon and disruptions in the *adc* gene, itself, a second phenotypic marker of phase variation was analysed; decreased sensitivity to erythromycin. Analysis indicated that the difference in hemolytic activity of MLT5 is probably the result of a disruption of *adc,* the adenylate cyclase toxin - hemolysin gene, which is in the same region as the Tn*5* insert in this mutant, as no corresponding increase in erythromycin resistance occurred to indicate a shift of the bacteria from virulence to avirulence. The absence of any significant difference in sensitivity to human sera between the hemolytic and nonhemolytic phenotypes of MLT5 also supports this hypothesis. Less drastic increases in production of nonhemolytic variants by MLT2 and MLT3 which are accompanied by an increase in erythromycin resistance will require more detailed analysis of the Tn*5* disrupted region for clarification of the situation but do implicate *B. pertussis* LOS in a previously unassociated phenomenon.

Studies of possible interactions between isolated LOS and associated molecules such as pertussis toxin have already been undertaken in several laboratories with encouraging results (29,35). Additional assays of biological alteration of whole cell properties are presently underway and should expand our understanding of the relationship of LOS structure to outer membrane function and bacterial pathogenicity. We are particularly interested in whether the endotoxic properties of the LOS mutant strains have been altered enough to possibly affect the local and systemic reactions associated with injection of the whole cell vaccine.

The bacterial outer membrane is the dynamic template upon which the host/pathogen relationship evolves. Endotoxin provides much of the "framework" for this matrix. The creation and characterization of these LOS mutants provides an opportunity by which the complete role of endotoxin in the pathogenicity and reactivity of the whole cell can be examined. Furthermore, these mutants provide a background in which LOS associations with other molecules can be studied. Secondary mutations in mutant strains could be used to define the nature of synergistic or associative relationships the LOS may have in vivo with other cell components such as pertussis toxin. Such interactions may be more important to in vivo infection and immunity than previously envisioned.

TABLE III.1: Reactivity of LOSA-specific monoclonal antibody BL-2 and LOSB-specific monoclonal antibody BL-8 to *B. pertussis* standard strains and LOS mutant strains MLT1 - 10.

SDS-PAGE LOS TYPE	BACTERIAL STRAIN	ISOLATED LOS		INTACT BACTERIAL CELL ASSAYS					
		Western Blot[a]		Dot Blot[a]		^{125}I-mAb Binding[b]		Complement Fixation[c]	
		BL-2 (LOSA)	BL-8 (LOSB)	BL-2 (LOSA)	BL-8 (LOSB)	BL-2 (LOSA)	BL-8 (LOSB)	BL-2 (LOSA)	BL-8 (LOSB)
A,B									
(A>>>B)	BP347	++++	+	+++	-	ND	ND	ND	ND
(A>>>B)	BP338	++++	-	+++	-	ND	ND	++	-
(A>>>B)	Tohama1	ND	ND	ND	ND	+++	+	ND	ND
(A>>B)	MLT3	+++	+/-[d]	+++	-	+++	+++	++	-
(A=B)	MLT1	+++	+++	+++	+++	+++	+++	++	+
A,B,IntA									
(B,IntA>>A)	MLT2	+	+++	+++	+	+++	+++	+/-	+
(B=IntA=A)	MLT4	+++	+	+++	-	+++	+++	++	+/-
(A>B>IntA)	MLT5	++	+	+++	-	+++	+	++	+/-
(A>>B,IntA)	MLT9	+++	+	+++	+	+++	++	+	-
A,B,IntB									
(A>B,IntB)	MLT8	+++	+	+++	+	+++	++	++	+/-
B									
(B)	MDH134-	-	++++	-	+++	+	+++	ND	ND
(B)	MDH134+	-	++++	-	+++	+	+++	-	++
B,IntA									
(IntA=B)	MLT6	-	+++	-	+++	+	+++	-	++
(IntA=B)	MLT10	-	+++	-	+++	+	+++	-	+
B,IntB									
(IntB=B)	MLT7	-	++	-	+++	+	+++	-	++

TABLE III.1 continued)

a: Western blot,dot immunoassay (by colour intensity):

++++ : very strong reaction

+++ : strong reaction;

++ : moderate reaction;

+ : slight reaction;

- : no reaction.

b: Radioimmunobinding : +++: >10000 cpm;

++: 5000 - 10000 cpm;

+: 1000 - 5000 cpm;

-: 0 - 1000 cpm.

c: Complement Fixation : +++: 80 - 100 % of available complement fixed;

++: 60 - 80 % of available complement fixed;

+: 40 - 60 % of available complement fixed;

+/-: 20 - 40 % of available complement fixed;

-: 0 - 20 % of available complement fixed.

d: +/- denotes a reactivity which is clearly visible upon extreme development of the blot in substrate buffer.

TABLE III.2: Growth And LOS Yield Of *B. pertussis* LOS Mutant And Standard Strains.

STRAIN	PHENOTYPE (SDS-PAGE, WESTERN BLOT)	BACTERIAL YIELD in g (WET WEIGHT / L CULTURE)	LOS YIELD in g (DRY WEIGHT / L CULTURE)	% WEIGHT IN COMPARISON WITH BP338 (parent strain)
MDH134+	LOSB	10.236	0.008	39.10 %
BP338	LOSA > LOSB	9.135	0.021	100.0 %
BP347	LOSA > LOSB	12.264	0.018	85.71 %
MLT1	LOSA = LOSB	12.153	0.023	109.5 %
MLT2	LOSA < IntA= LOSB	9.407	0.017	80.95 %
MLT4	LOSA = IntA = LOSB	11.009	0.016	76.19 %
MLT5	LOSA > IntA = LOSB	9.773	0.017	80.95 %
MLT7	IntB = LOSB	10.672	0.003	14.29 %
MLT10	IntA = LOSB	9.438	0.004	19.05 %

TABLE III.3: Pulsed-field gel analysis and mapping of Tn*5* insertions in LOS mutants MLT 1 - 10 to the parental *B. pertussis* strain (BP338) genome.

SDS-PAGE LOS TYPE	STRAIN	RESTRICTION ENZYME		GENOME POSITION in 3750 kb Genome
		*Xba*I Tn*5* Band Location on PFGE map	*Spe*I Tn*5* Band Location on PFGE map	
A,B				
	MLT1	A	C	3000-3200kb
	MLT3	L4	C	3200-3300kb
A,B,IntA				
	MLT2	A	J2	3200-3300kb
	MLT4	A	C	3050-3200kb
	MLT5	E	E1	60-300kb
	MLT9	A	J2	3200-3300kb
A,B,IntB				
	MLT8	H	E3	2750kb-2820kb
B,IntA				
	MLT6	A	J2	3200-3300kb
	MLT10	A	J2	3200-3300kb
B,IntB				
	MLT7	A	J2	3200-3300kb

Position of Tn*5* insert on restriction-digested pulse-field gel as determined by *Xba*I and *Spe*I digest. Position of mutants on BP338 genome as mapped by Stibitz et al (37).

TABLE III.4: Notable[a] Alterations in Antibiotic Sensitivity Of *Bordetella pertussis* LOS Mutant Strains

BACTERIAL STRAINS	ANTIBIOTIC SENSITIVITY (MIC in µg/ml)							
	Increase in Sensitivity	Decrease in Sensitivity				Tn5-Related Decrease in Sensitivity		
	Novobiocin	Oleic Acid	Sulpha-methoxazole	Tetracycline	Trimethoprim	Amikacin	Kanamycin	Streptomycin
Control Strains								
BP338	0.16	0.12	4.00	0.20	4.00	16.0	8.00	15.0
BP347	0.03	1.00	0.25	1.60	16.0	96.0	>32.0	>500
MDH134+	0.16	0.12	0.50	0.20	1.00	8.00	4.00	5.00
Mutant Strains								
MLT1	0.02	0.25	4.00	0.80	4.00	182	>32.0	>500
MLT2	0.02	0.50	16.0	0.60	16.0	144	>32.0	>500
MLT4	0.02	0.50	16.0	0.80	16.0	182	>32.0	>500
MLT7	0.03	0.50	16.0	0.80	24.0	182	>32.0	>500
MLT10	0.16	0.25	16.0	0.80	16.0	182	>32.0	>500

a) "Notable" refers to a difference of at least 2 twofold dilutions in at least one of the mutants when compared with the parental strain in 2 separate assays (each strain tested in triplicate/assay).

TABLE III.5: Slight[a] Alterations in Antibiotic Sensitivity Of *Bordetella pertussis* LOS Mutant Strains

BACTERIAL STRAINS	ANTIBIOTIC SENSITIVITY (MIC in µg/ml)								
	Increase in Sensitivity					Decrease in Sensitivity			
	Cefotaxime	Cephalothin	EDTA	EGTA	Vancomycin	Ampicillin	Erythromycin	Fusidic Acid	Rifampicin
Control Strains									
BP338	1.00	4.00	0.16 mM	1.25 mM	24.0	5.00	0.02	0.06	0.06
BP347	4.00	20.0	0.16 mM	1.25 mM	256	30.0	0.12	0.25	0.50
MDH134+	0.25	2.00	0.16 mM	0.63 mM	8.00	2.50	0.02	0.04	0.03
Mutant Strains									
MLT1	0.50	2.00	0.08 mM	0.63 mM	24.0	30.0	0.02	0.06	0.06
MLT2	0.50	4.00	0.08 mM	0.63 mM	16.0	30.0	0.06	0.06	0.12
MLT4	0.50	2.50	0.08 mM	0.63 mM	16.0	30.0	0.06	0.09	0.12
MLT7	0.50	1.50	0.08 mM	0.63 mM	16.0	15.0	0.06	0.12	0.12
MLT10	0.50	2.50	0.08 mM	0.63 mM	16.0	15.0	0.06	0.12	0.12

a) "Slight " refers to a difference of less than 2 twofold dilutions in at least one of the mutants when compared with the parental strain in 2 separate assays (each strain tested in triplicate/assay).

FIGURE III.1

Silver-stained SDS-PAGE profile of lipooligosaccharide from proteinase K-treated Laemmli digests of *B. pertussis* standard strains and Tn*5*-insert LOS mutants MLT 1-10.

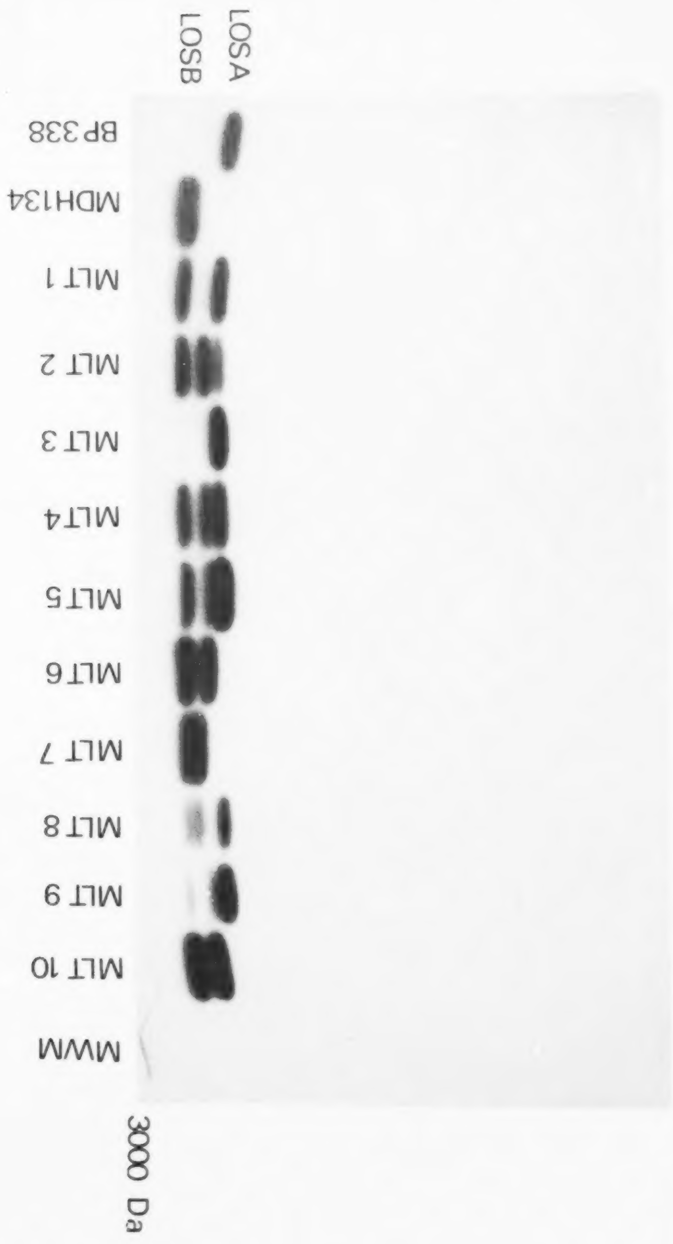
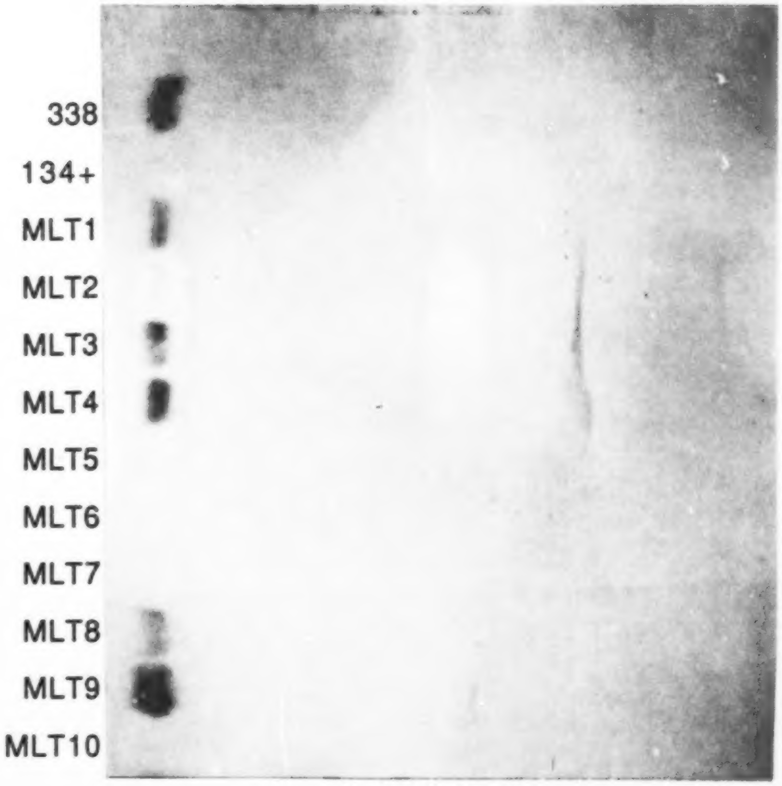

LOSA
LOSB
BP338
MDH134
MLT 1
MLT 2
MLT 3
MLT4
MLT5
MLT6
MLT 7
MLT 8
MLT 9
MLT 10
MWM
3000 Da

FIGURE III.2

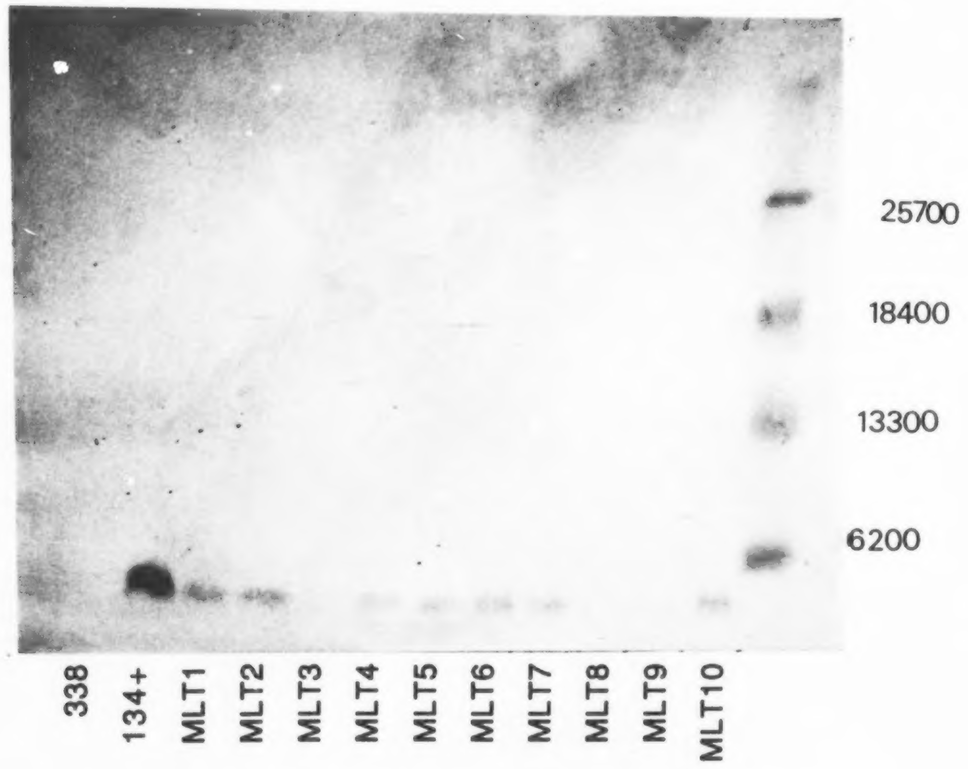
Western immunoblot assay of reactivity of LOS-specific monoclonal antibodies with SDS-PAGE of proteinase K - treated Laemmli digests of *B. pertussis* standard strains and Tn*5*-insert LOS mutants MLT 1-10.

a) Reactivity of LOSA-specific monoclonal antibody BL-2 with SDS-PAGE processed PK-Laemmli digests of *B. pertussis* standards and mutants.

b) Reactivity of LOSB-specific monoclonal antibody BL-8 with SDS-PAGE processed PK-Laemmli digests of *B. pertussis* standards and mutants.



B. PERTUSSIS CONTROL AND MUTANT STRAINS



B. PERTUSSIS CONTROL AND MUTANT STRAINS

FIGURE III.3

^{125}I - Radioimmunobinding assay of accessibility of whole *B. pertussis* mutant and standard organisms to LOSA and LOSB specific monoclonal antibodies, BL-2 and BL-8, respectively. Bars represent standard deviation among the means of three independant assays.

Figure 3

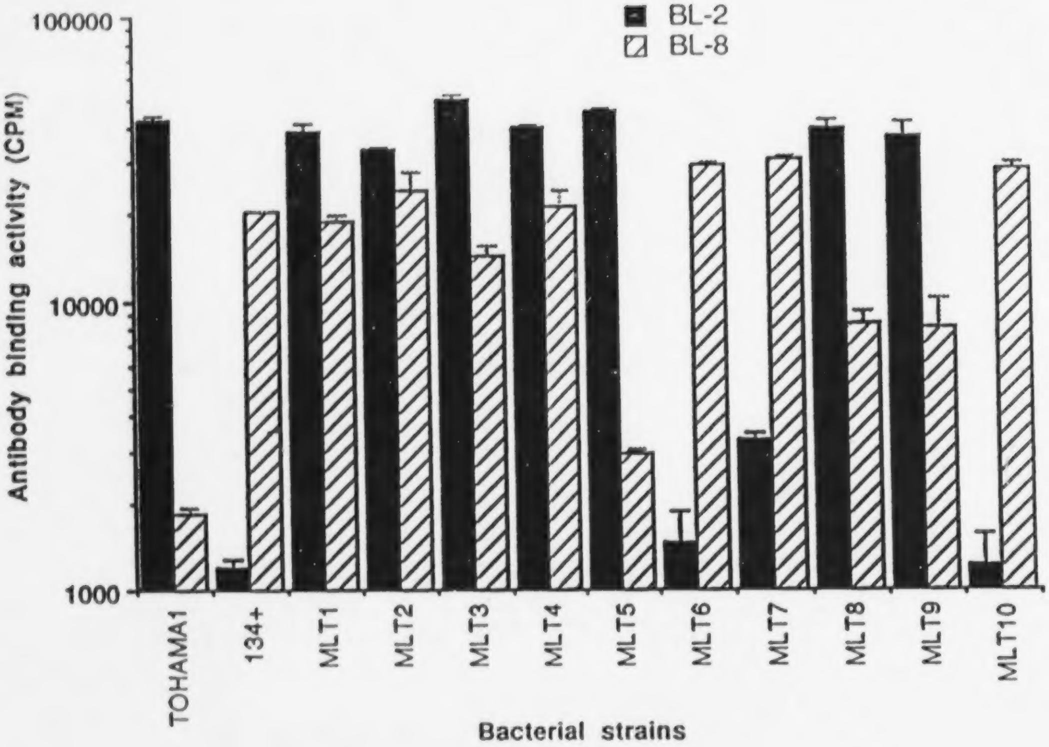

■ BL-2
▨ BL-8
Antibody binding activity (CPM)
100000
10000
1000
TOHAMA1
134+
MLT1
MLT2
MLT3
MLT4
MLT5
MLT6
MLT7
MLT8
MLT9
MLT10
Bacterial strains

FIGURE III.4

Autoradiographs of southern blot analysis of band location of ^{32}P-labelled Tn*5* probe in *B. pertussis* LOS mutants restricted with; **A)** *Eco*R1, **B)** *Cla*1, **C)** *Sma*1, **D)** *Bam*H1, and **E)** *Sal*1. Molecular weight markers are shown as follows (in kb):

a) 9.4, b) 6.6, c) 4.4, d) 2.3, e) 2.0.

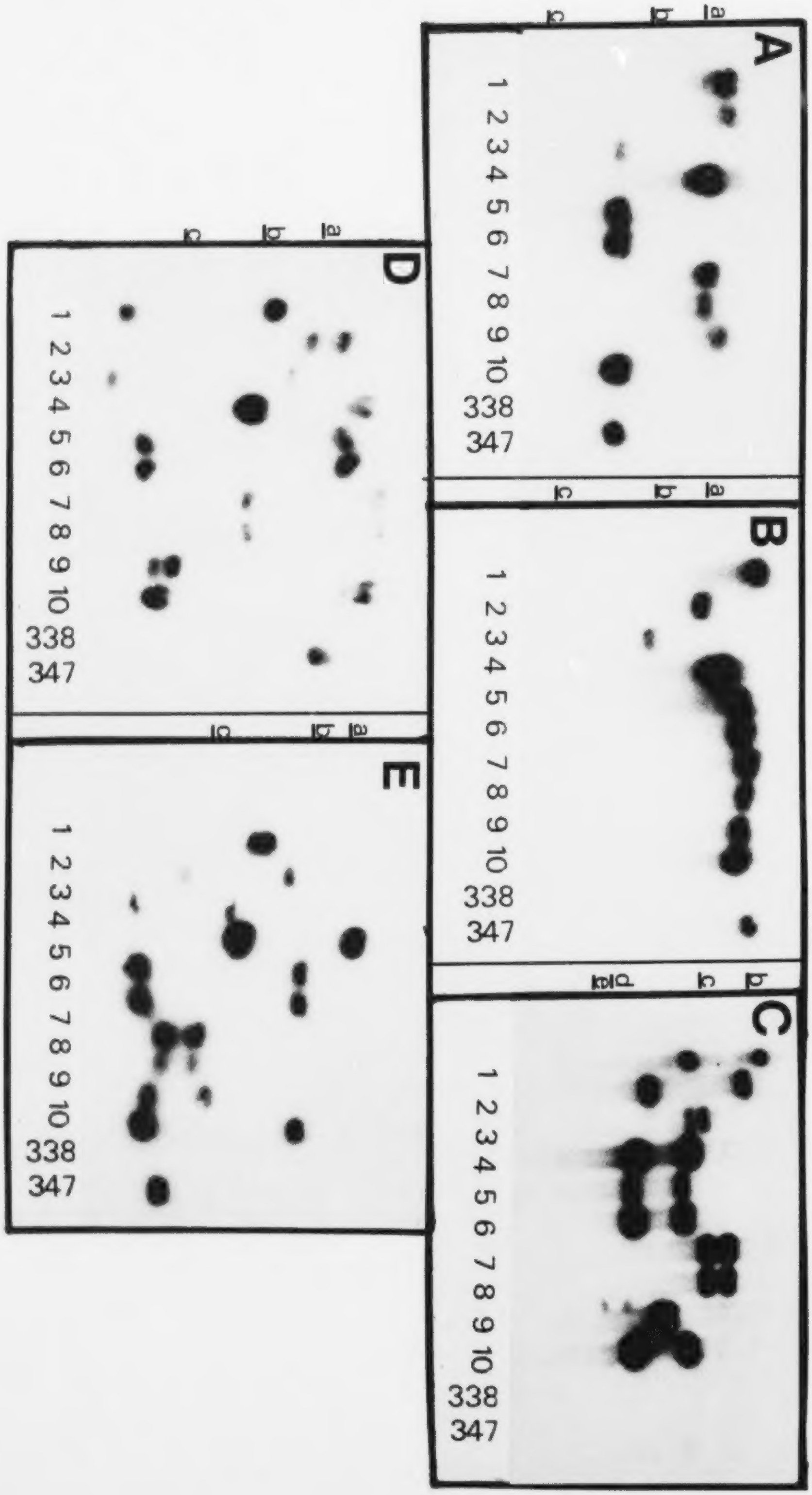

A
a
b
c
1 2 3 4 5 6 7 8 9 10 338 347
B
a
b
c
1 2 3 4 5 6 7 8 9 10 338 347
C
b
c
d
e
1 2 3 4 5 6 7 8 9 10 338 347
D
a
b
c
1 2 3 4 5 6 7 8 9 10 338 347
E
a
b
c
1 2 3 4 5 6 7 8 9 10 338 347

FIGURE III.5

Location on *B. pertussis* genomic map of Tn*5* inserts in LOS mutants and genes for pertussis toxin (*ptx*), dermonecrotic toxin (*dnt*), and adenylate cyclase (*cya*) and *Bordetella* virulence genes A and S (*bvgA, bvgS*)(37).

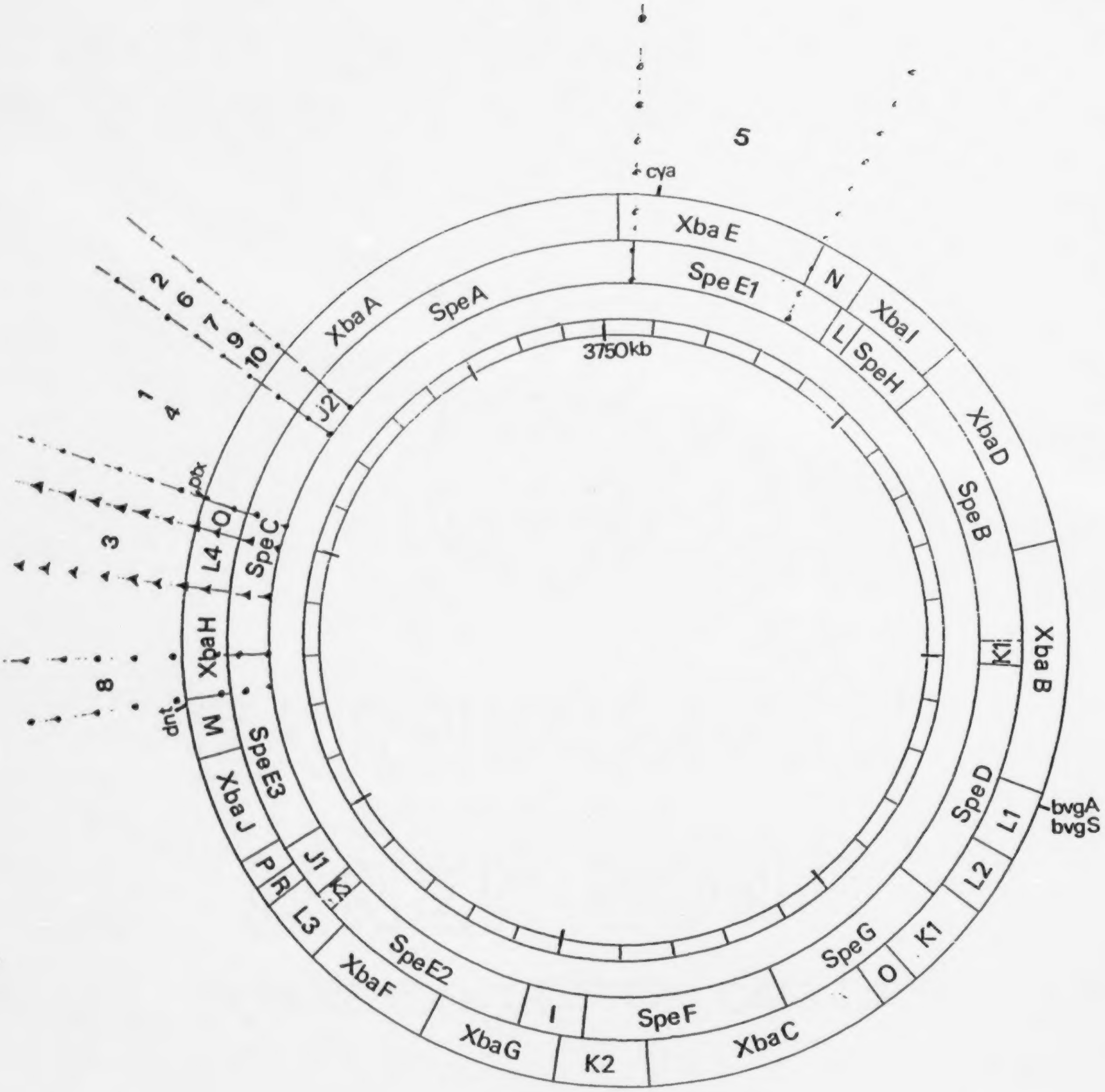

5
cya
Xba E
Spe E1
N
Xba I
L
SpeH
XbaD
SpeB
Xba B
K1i
SpeD
L1
bvgA
bvgS
L2
K1
O
SpeG
XbaC
SpeF
K2
I
XbaG
XbaF
SpeE2
L3
R
K2
J1
P
XbaJ
SpeE3
M
dnt
XbaH
8
L4
SpeC
O
ptx
3
1
4
2
6
7
9
10
J2
XbaA
SpeA
3750kb

FIGURE III.6

Complement fixing ability of *B. pertussis* LOS mutant and standard organisms in conjunction with LOSA and LOSB-specific monoclonal antibodies, BL-2 and BL-8, respectively. Hemolysis of sensitized red blood cells (sRBCs) was measured spectrophotometrically by analysis of freed oxyhemoglobin at an absorbance of 540 nm. The extent of complement fixation was measured as the total complement available (100%) minus the percentage of complement which remained free (after incubation with the mAb and bacteria) to lyse sRBCs. Bars indicate standard deviation of three independant assays done in quadruplicate.

Figure 6

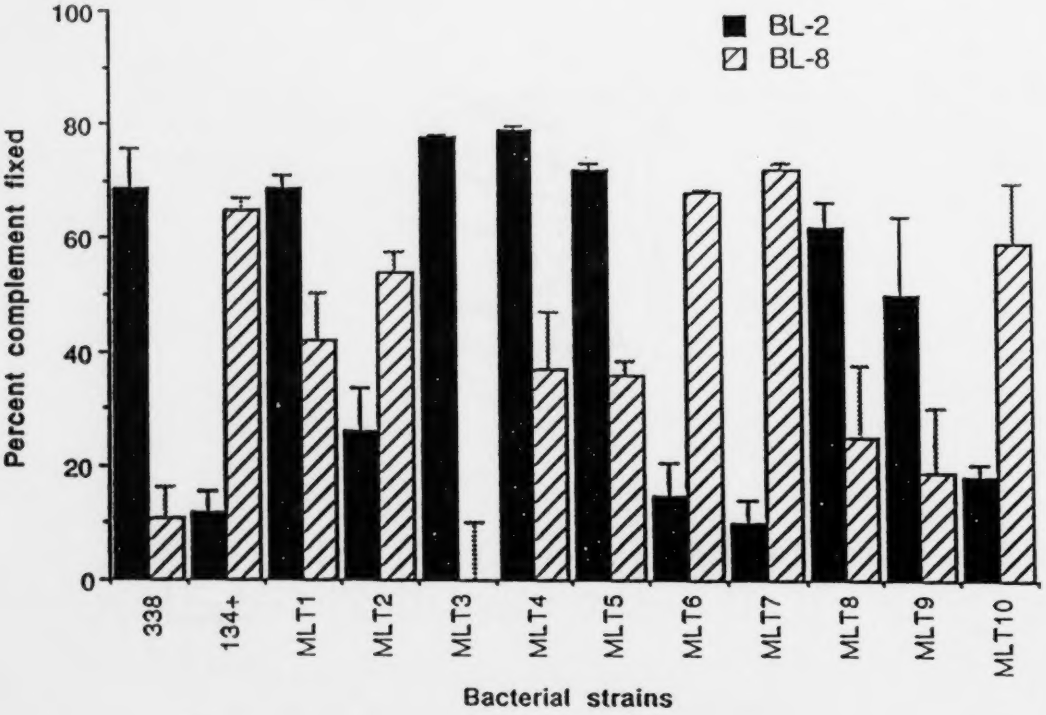
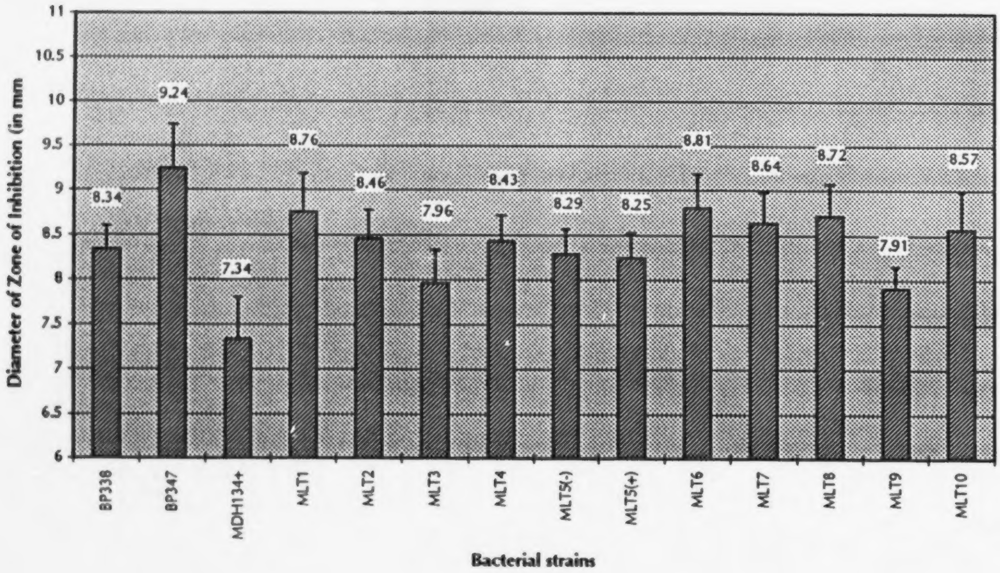

■ BL-2
▨ BL-8
Percent complement fixed
100
80
60
40
20
0
338
134+
MLT1
MLT2
MLT3
MLT4
MLT5
MLT6
MLT7
MLT8
MLT9
MLT10
Bacterial strains

FIGURE III.7

Sensitivity of *B. pertussis* LOS mutant and standard organisms to pooled normal human sera. Bars indicate standard deviation amongst results of 4 independant experiments done in pentuplicate; that is each sample was tested 5 times for each of 4 independent experiments. MLT5(+) and MLT5(-) are respectively virulent and avirulent phenotypes of MLT5. p<.01 by student t-test for BP338 vs. BP347, 134+ and all LOS mutant strains save MLT2 (p=.2173), MLT4 (p=.3383), MLT5 (p=.2845). p<.01 by student t-test for 134+ vs. BP338, BP347 and all mutant strains.



Sensitivity of *Bordetella pertussis* control and mutant strains to killing by normal human serum.

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CHAPTER IV

Creation, Selection And Characterization Of *Bordetella bronchiseptica* Mutants With Tn*5*-Induced Alterations In Lipopolysaccharide.

A. INTRODUCTION

Bordetella bronchiseptica is a Gram-negative pathogen responsible for upper respiratory tract infections in numerous animal species such as dogs, cats, rabbits and guinea pigs. The bacterium is of significant economic and agricultural importance as a conditioning factor in the development and spread of swine atrophic rhinitis, and rabbit tracheal bronchitis (16,44). As well, *B. bronchiseptica* is the causative agent of canine kennel cough and has also been identified as a rare cause of human whooping cough. In immunocompromised patients the organism has been known to result in pneumonia and septicemia. This has become a source of greater medical concern with the onset of AIDS (44).

Unlike *Bordetella pertussis*, the main agent responsible for human pertussis, *B. bronchiseptica* is a nonfastidious organism which is motile and can be found in many environmental niches (16,28,44). Although capable of producing many of the factors associated with *B. pertussis* pathogenicity, the decreased virulence of *B. bronchiseptica* in humans has often been attributed to it's lack of pertussis toxin. This multifunctional toxin is encoded by all *Bordetellae* but is produced by *B. pertussis*, alone (16,28,31,41,44). The lack of this protein, however, does not explain the many differences in outer membrane function which differentiate *B.*

bronchiseptica from the other *Bordetellae,* most notably greatly increased resistance to killing by human serum as well as numerous antibiotics.

Numerous studies have documented the role of Gram negative endotoxin in the barrier and molecular exclusion activities of the outer membrane which govern these resistances. Genetic truncation of the O-unit of *Salmonella minnesota* lipopolysaccharide results in drastic alterations in sensitivity to a number of antibiotics as well as normal human serum. The endotoxins of *B. bronchiseptica* and *B. pertussis* are structurally very similar. The core of *B. bronchiseptica* LPS has a high degree of structural homology with the core structure of *B. pertussis* endotoxin (8,10). The endotoxins, however, differ significantly in the presence of an O-chain polysaccharide moiety in *B. bronchiseptica* LPS which *B. pertussis* lacks. Another important distinction between the two species is the fact that while the endotoxin of *B. pertussis* remains unchanged the LPS structure of *B. bronchiseptica* varies with virulence (9,27). In virulent *B. bronchiseptica* organisms the LPS presents on SDS-PAGE as two low molecular weight bands, termed LPSA and LPSC, with a much higher O-chain sugar "ladder". In avirulent organisms the O-chain ladder remains constant, however, the low molecular weight bands are even lower and have been termed LPSB and LPSD, respective of decreasing molecular size. The significance of this variation to virulence has not been determined. Several studies, however, have demonstrated the isolated lipopolysaccharide (LPS) of *B. bronchiseptica* to be as toxic as that of *B. pertussis* and enteric species in assays of pyrogenicity, TNF induction, mitogenicity as well as numerous other properties (21,38).

The structural and functional similarities of many of the virulence factors of the *Bordetellae* have been established though the isolation and characterization of the individual molecules for each species (39,40). In an effort to better understand the role *B. bronchiseptica* LPS plays as a component of the outer membrane our laboratory has created and characterized 26 *B. bronchiseptica* Tn*5* insertion mutants with LPS alterations. Five different phenotypes were established and representative mutants from each subset were chosen for further investigation of the nature of these changes and their effect on certain functions of the intact cell. The presence of an O-unit constitutes the only documented structural difference between the LPS of *B. bronchiseptica* and the LOS of *B. pertussis*. Thus, the effect of the LPS truncations on outer membrane functions of *B. bronchiseptica* was studied in an attempt to determine the role played by the O-unit and the endotoxin in membrane-associated functions; notably the large differences observed in these functions between *B. pertussis* and *B. bronchiseptica*.

Analysis with monoclonal antibodies (mAb) reactive with the parental strain demonstrated that the LPS mutants characterized had alterations in outer membrane epitope exposure and receptor functions. Genetic analysis confirmed the presence of a single Tn*5* insert and indicated unique insertion sites in each of the mutant genomes. Alterations in the LPS mutants to the bactericidal activity of the D13B11 mAb and increased sensitivity to killing by normal human serum demonstrated disruptions in the important immune functions governed by the outer membrane. Sensitivity of the LPS mutants, however, to numerous antibiotics was largely unaffected by the LPS truncations indicating that the O-unit and likely the LPS, in general, is not the structure directly responsible for the increase in antibiotic sensitivity noted in *B. pertussis* when compared with *B. bronchiseptica.*

B. MATERIALS AND METHODS

Strains and monoclonal antibodies

Strains used in this study were *Bordetella bronchiseptica* strains 110H+ (virulent) and 110H- (avirulent) which were obtained from D. Bemis (University of Tennessee, Knoxville, Tennessee, USA). *Bordetella pertussis* strain BP347, was obtained from A. Weiss (University of Cincinnati, Cincinnati, Ohio, USA). BP347 is the avirulent Tn*5* mutant which was produced by A. Weiss from BP338, which in turn was selected as a spontaneous nalidixic acid mutant of Tohama 1. The Tn*5*-containing vector pUW964, was constructed and provided by A. Weiss (University of Cincinnati, Cincinnati, Ohio, USA (39,40)).

. The *B. bronchiseptica* strains 110H+ and 110H- were maintained on Bordet-Gengou Agar (BGA). The avirulent strain was chosen as the LPS bands were lower in molecular weight than those of 110H+ and the most extensive LPS truncation possible was desired in mutants. The parental strain was selected by plating 110H- on BGA plates containing 60 μg/ml nalidixic acid. The resulting nalidixic acid resistant strain was deemed 110H-n and was maintained on BGA plates containing 60 µg/ml nalidixic acid. Tn*5*-insertion mutants were grown on BGA containing 60 µg/ml nalidixic acid and 25 µg/ml kanamycin. *E. coli* strain HB101 containing the Tn*5* plasmid, pUW964, was maintained on L-agar containing 25 µg/ml kanamycin (39).

B. pertussis LOSA specific monoclonal antibodies (mAbs), 14A8 and 1H2, as well as *B. parapertussis* O-chain specific mAbs, D13B11 and A7D12, were developed and characterized in our laboratory (Peppler et al., manuscript in preparation). The *B. pertussis* LOSA specific mAb, BL-2, and the *B. pertussis* LOSB specific mAb, BL-8, were provided to us by D. Martin (Unite de recherche en vaccinologie, Croix-Rouge/Recherche et developement, Ste-Foy, Quebec) (Refer to Chapter IV for details of specificity (2,6,23)). Ascites fluid containing the mAbs were stored at -20°C, then thawed and diluted in blocking buffer (3% bovine serum albumin in 50mM tris-buffered saline, pH 7.0 (TBS)) for experiments.

***B. bronchiseptica* growth in broth and LPS extraction.**

Initial 100 ml cultures of all *B. bronchiseptica* strains were inoculated from 2 day cultures harvested from BGA plates. These cultures were grown with aeration overnight at 37°C in Stainer-Scholte medium with 10 grams/L added casamino acids. The "seed" cultures were then used to inoculate 900 ml of the same media and the broth was incubated for a further 2 days at 37°C with aeration. The LPS was extracted by the standard hot phenol/water method of Westphal and

Jann commonly used for *B. bronchiseptica* LPS purification as detailed in Chapter II (27,42,50). Protein content was analyzed by Coomassie and silver stained SDS-PAGE gels.

Mutation of the wild-type *B. bronchiseptica* genome by random insertion of a Tn*5* transposon.

The nalidixic acid resistant variant 110H-n was mutated by random insertion of a Tn*5* transposon. The transposon was transferred though conjugation of the parental 110H-n strain with an HB101 *E. coli* strain containing a Tn*5* suicide vector, pUW964 (39). *B. bronchiseptica* strain 110H-n was grown overnight on BGA containing 60 µg/ml nalidixic acid. *E. coli* strain HB101 containing the pUW964 suicide plasmid was grown overnight on L-agar containing 25 µg/ml of kanamycin. Each organism was swabbed from its growth plate (normal BGA with required antibiotics) onto a mating plate (BGA containing either 10 mM $MgCl_2$ or 10 mM $MgSO_4$) and incubated at 37°C for 2.0 h. After incubation the organisms were swabbed from the mating plate to 2.0 ml of Stainer-Scholte broth. Mutants were selected by plating 100µl aliquots of a 10^{-1} dilution of this suspension onto BGA plates containing both 25 µg/ml of kanamycin and 60 µg/ml of nalidixic acid. This protocol routinely resulted in 100-300 kanamycin-resistant, nalidixic acid-resistant *B. bronchiseptica* colonies per plate after 2 days growth at 37°C.

Western Colony Blot Analysis

Putative LPS mutants were screened by western colony blot with the *B. parapertussis* O-chain specific monoclonal antibody D13B11. As the O-units of *B. parapertussis* and *B. bronchiseptica* are chemically identical this monoclonal antibody reacts with the LPS O-chains of *B. bronchiseptica*. Western colony blot analysis was performed as detailed in Chapter II.

The primary antibody (Ab), D13B11, was added to the blocking buffer at a dilution of 1:100. Colonies which did not react with the anti-O-chain mAb, D13B11, were selected from the original plates, subcultured, and their LPS profiles confirmed by SDS-PAGE, silver stain, Western blot and whole cell ELISA analysis.

SDS-PAGE, Silver Stain, and Western Blot Analysis.

Lipopolysaccharide was visualized from *B. bronchiseptica* whole-cell lysates by solubilization of bacteria suspended in 50 mM Tris-glutamate saline at pH 7.5 (TGS) to an optical density of 0.3 measured at a wavelength of 540 nm. The method of Laemmli et al. was used as detailed in Chapter II (20,27).

SDS-PAGE and Western blot analysis was performed as specified in Chapter II. Silver stain was used to visualize LPS on SDS-PAGE gels. For Western blots the primary antibody (mAbs D13B11, A7D12, 1H2, 14A8, BL-2) was added to the blocking buffer at a dilution of 1:100.

Whole Cell ELISA

Bacteria were suspended from BGA plates to an optical density of 0.20 at an absorbance of 540 nm (A_{540}) in PBS containing 0.1% Thimerosal. The whole cell ELISA assay was performed using the procedure detailed in Chapter II. Overnight cultures were used to coat the ELISA plates.

Southern Blot Analysis

To determine which bands contained the Tn*5* insertion, Southern hybridizations were done with a Tn*5* transposon which was labelled with ^{32}P using the random primer labeling kit available from Gibco/BRL (Burlington, Ontario). Genomic *B. bronchiseptica* DNA was isolated by the CTAB method (3). DNA was restricted for 2 h at 37°C with four separate restriction enzymes; *Bam*H1, *Cla*1, *Eco*R1, and *Sal*1. Digests were run on 0.6% agarose gels at 100 volts for 3 h. Southern analysis was carried out as detailed in Chapter II (32).

Radial Diffusion Immunoassay for Assaying Bactericidal Activity and Sensitivity to Human Serum

The method of Fernandez and Weiss was used (personal communication) as specified in Chapter II. Overnight plate cultures of *B. bronchiseptica* were utilized in theassay. For determination of bactericidal activity 5 µl of 50% guinea pig serum/50% mAb D13B11 was added to each well. The complement in guinea pig sera could not, alone, lyse *B. bronchiseptica* cells.

Antibiotic Sensitivity Assay

Strains were grown overnight on BGA then suspended in 4 ml of SSB to an optical density of 0.20 at a wavelength of 540 nm (27). The suspension was then diluted and plated in duplicate to BGA plates to determine the total viable count of organisms used in the inoculum. The agents were selected based on previous studies on the effects of various antibiotics on *Bordetellae* species and enteric LPS (4,5,31,33,35). Antibiotic sensitivity assays were performed as stipulated in Chapter II. The plates were incubated for 2 days at 37°C. Minimum Inhibitory Concentration (MIC) was measured as the last clear well (no turbidity) both by eye and by ELISA reader at an absorbance of 540 nm. Final MIC data was based on geometric mean titres.

C. RESULTS

General characteristics of LPS mutants.

All LPS mutant strains demonstrated a colonial morphology which was phenotypically similar to the parent strain, 110H-n. Growth rate, likewise, was unaffected by the presence of the Tn*5* insert; colonies of approximately 2 to 3 mm. in diameter were produced within 24 h and the lack of hemolytic activity indicated a retention of parental avirulence. As well, broth cultures of mutant strains grew at the same rate as the parental strain.

Tn*5* mutants with altered LPS were negatively selected from the mutant population (nalidixic acid and kanamycin - resistant colonies) using D13B11, a monoclonal antibody which recognizes a presently undetermined epitope on the O-chains of both *B. parapertussis* and *B. bronchiseptica*. A lack of reactivity of colonies to this mAb indicated a modification of the LPS O-chain. The LPS was isolated from such colonies and further analysed by SDS-PAGE. Coomassie and silver stained SDS-PAGE gels of whole cell lysates indicated no notable alteration in protein content in any of the selected mutants.

Analysis Of Phenotypic Alterations In The Profiles Of Isolated LPS Of Tn*5* Mutants.

Mobility in SDS-PAGE.

The SDS-PAGE silver stain data indicated the presence of four major phenotypic subsets amongst the 26 LPS mutants isolated (Figure IV.1A). None of the mutant strains possessed evident O-side chain "ladders" by silver stain analysis therefore the mutants were grouped according to similarities in the lower core bands. One group possessed prominent LPSB and LPSD bands, similar to the parental strain, 110H-n. A second subset contained a prominent LPSB band and little LPSD while the third grouping consisted of mutants with a prominent LPSD band and little or no LPSB. The final set was composed of a single mutant, Bbs-12, whose LPS was composed of a single band that was distinctly lower in relative mobility than LPSD.

Western Blot Analysis.

The initial number of phenotypic subsets of LPS mutation found in the *B. bronchiseptica* strains was expanded by analysis of the reactivity of mutant LPS with monoclonal antibodies to various epitopes on *B. pertussis* and *B. parapertussis* endotoxin (Figure IV.1B). Five distinct phenotypes were distinguished by comparison of the SDS-PAGE profiles and antibody reactivity patterns. Reactivity of the various monoclonal antibodies with isolated LPS was analagous to results obtained with intact cells as analysed by whole cell ELISA (data not shown). The final subsets are summarized in Table IV.1 and possess the following characterisitics:

1) LPS with LPSB, LPSD and altered O-side chain.

The major difference between the LPS of these mutants and the parental phenotype was the presence of an altered O-side chain. This alteration was notable in colony blot assays and western blot data of isolated LPS reacted with the cross-reactive *B. parapertussis* O-chain mAb D13B11 (Figure IV.1B). Only a few bands were visible as opposed to the full ladder of the LPS of the parent strain, 110H-n (Figure IV.1B). The SDS-PAGE profiles, however, indicated a total absence of the O-chain moiety (Figure IV.1A).

2a) LPS with LPSB, LPSD and no O-chain.

This phenotype encompassed the largest group of mutants. All assays indicated a complete lack of O-chain material while retaining LPSB and LPSD bands in quantities concordant with the parental phenotype (Figure IV.1A). The absence of O-unit indicated by silver stain was confirmed by the lack of reactivity of mutant LPS with either of the cross-reactive *B. parapertussis* mAbs, D13B11 and A7D12 in western blots (Figure IV.1B) (TableIV.1). Mutant Bbs-1, however, was notable in its increased reactivity with the LOSA mAbs, BL-2 and 14A8. Bands too low to be conventional O-chain material yet slightly above the LPSB band were visible in this mutant where they were absent in the parent strain.

2b) LPS with increased LPSB and very little LPSD.

Just two mutants of the large phenotoypic subgroup which consisted of LPSB and LPSD but no O-unit contained LPSB as the major band, with a significantly decreased LPSD band. Bbs-2 and Bbs-5 displayed this notable variation in LPS band ratio (Figure IV.1A). Both mutants exhibited a decreased reactivity with the O-chain mAbs, D13B11 and A7D12, however, each was notable in the presence of slightly high molecular weight bands on SDS-PAGE gels as seen with Bbs-8 and Bbs-9 as well as with the LOSA mAbs, in the case of Bbs-2 (Figure IV.1B).

3) LPS with LPSB, only and complete O-chain.

Although Bbs-8 and Bbs-9 were missing an O-chain on silver stain SDS-PAGE gels (Figure IV.1A), O-chain material was clearly visible on western blots incubated with the D13B11mAb (Figure IV.1B). As well, some truncated O-unit or slightly "expanded" core material was visible as bands when the LPS from these two strains were incubated with the *B. pertussis* LOSA mAbs, BL-2 and 14A8 (Figure IV.1B).

4) LPS with increased LPSD and little, if any LPSB.

By silver stained SDS-PAGE analysis, mutants Bbs-6, Bbs-11, Bbs-13, and Bbs-21 contained little if any LPSB (Figure IV.1A). The LPS of these mutants did not react with any of the O-chain or *B. pertussis* LOSA band mAbs (Figure IV.1B).

5) LPS with a band lower than LPSD, only.

The sole constituent of this group, Bbs-12, had LPS which presented on silver stained SDS-PAGE as a single band of slightly lower molecular weight than LPSD (Figure IV.1A). Although the LPS of this mutant proved to be similar to the LPSD mutants in its antigenic unresponsiveness to most of the mAbs assayed, it differed in its reactivity with the O-chain mAb, D13B11 (Figure IV.1B). A diffuse band of low O-chain like material was noted at a molecular weight of approximately 13 kDA.

Alterations in Growth and Extraction of LPS in Control and LPS Mutant Strains.

Representative strains were chosen from the various groups. Bbs-14 and Bbs-25 were chosen to depict the variety of O-unit truncations which comprised Group#1. Bbs-9 was chosen to represent the antigenically altered O-unit phenotype of Group#3. Bbs-6 represented the LOSD phenotype of Group#4. The only deep rough LOSD- mutant, Bbs-12, characterized Group#5. Unfortunately Bbs-5 was chosen to represent Group#2 from initial data before greater elucidation of the bands on SDS-PAGE silver stained gels indicated the decrease in LOSB.

All mutants were grown in one litre broth culture and the LPS extracted by the standard hot phenol/ water protocol used for extraction of LPS from the parental strain, 110H-n. The LPS of those mutants lacking an extensive O-chain moiety extracted very poorly with this method. Although similar in bacterial growth, the yields of Bbs-6 and Bbs-12 LPS were lower than those of Bbs-5, Bbs-9, Bbs-14, Bbs-25, 110H-n, and 110H+ (Table IV.2). The extraction procedure was repeated twice with similar results, (the average +/- standard deviation). As well, the LPS of both Bbs-6 and Bbs-12 was extracted by the PCP (phenol-chloroform-petroleum ether) extraction protocol (13) and LPS yields did not notably improve.

Genetic analysis of Tn*5* location in genomic DNA of LPS mutant strains.

Southern Blot Analysis

The presence of a single Tn*5* insert in each of the mutant strains was confirmed by Southern analysis (Figure IV.2). The Tn*5* probe hybridized with a single band in mutant and control strains when DNA was digested with enzymes such as *Cla*1 and *Eco*R1 which do not recognize sites within the Tn*5* gene and thus produce only one hybridizable fragment. When digested with enzymes such as *Bam*H1, and *Sal*1 which have a single cut site within the Tn*5* gene each DNA digest produced two bands which hybridized with the Tn*5* probe. The lack of two distinct bands with digests of BP347, Bbs-25, Bbs-5 and Bbs-12 (for *Sal*1 digest, only) is likely due to a similarity in size of the two restriction fragments and the poor resolution of small differences in such large fragments when using a 0.6% agarose gel system.

Analysis of LPS Alteration on Bacterial Outer Membrane Function

Alterations in the Bactericidal Activity of D13B11 mAb in the Presence of Guinea Pig Complement.

The parental strain, 110H-n, the virulent control strain 110H+, and each of the Tn*5* mutant strains were assayed for sensitivity to complement killing mediated by the O-chain reactive *B. parapertussis* mAb, D13B11 (Figure IV.3). In this agar immunodiffusion assay system neither the virulent control strain 110H+, the parental strain 110H-n, nor the LPSD mutant Bbs-6 showed any susceptibility to classical pathway killing as mediated by D13B11. These results were consistent over four trials each done in pentuplicate. This differs from assays done in this laboratory wherebye 110H+ and 110H- were incubated with D13B11 and complement in broth then plated for viable counts (Peppler et al, manuscript inpreparation). This broth incubation/plate count protocol indicated a susceptibility for 110H+ (but not for 110H-, the parent strain of 110H-n). The most susceptible mutant to D13B11 mediated complement killing, Bbs-12 (with a approximate 5 fold increase in sensitivity), had the most extremely truncated LPS molecule. Bbs-9, the next most sensitive strain (approximate 4.5 fold difference), has an extended O-chain structure but is lacking the LPSD band. The remaining strains, except Bbs-6, all showed susceptibility to the bactericidal activity of D13B11 in concert with guinea pig complement at a slightly reduced level in comparison with Bbs-12 and Bbs-9 (approximate 4 fold increase in sensitivity).

Alteration in Sensitivity of LPS Mutants to Normal Human Serum.

The LPS mutant strains were approximately 400% more susceptible to complement-mediated killing by normal pooled human serum than were the control strains, 110H-n and 110H+ (Figure IV.4). The range extended from 3.96 fold (Bbs-9) to 4.54 fold (Bbs-25). As well, whereas all of the mutants, except Bbs-6, were slightly sensitive to undiluted guinea pig sera, alone, in four assays neither Bbs-6 nor the control strains showed any susceptibility to killing mediated by normal guinea pig sera (data not shown).

Alteration in Sensitivity of LPS Mutants to Antibiotics and Membrane-Active Agents.

In broth dilution assays the control strains, as well as the LPS mutants, were all exposed to a number of agents such as antibiotics, detergents and cation chelators whose interactions with the outer membrane have been correlated with LPS structure and function (17,18,26,37). Although neither the mutants nor the control strains could be considered sensitive to any of the agents assayed, relative differences between the parent and mutant strains were consistent with a number of antibiotics (Table IV.3).

As initial screening of LPS mutants was for the kanamycin resistance encoded by the Tn*5* insert it is not suprising that the mutants exhibited increased resistance to a structurally similar compound such as amikacin. Streptomycin resistance is an unexplained but documented consequence of Tn*5* insertion (39). No differences were noted in the sensitivity of the mutants to ampicillin, cefotaxime, chloramphenicol, fusidic acid, gentamycin, imipenem, novobiocin, oleic acid, oxacillin, polymyxin B, vancomycin or EDTA.

Notable differences were defined as differences of 2 or more dilution factors of 2. Slight differences were defined as changes of less than 2 twofold dilutions.

A notable increase was observed in sensitivity to ticaricillin, alone. Slight increases in sensitivity were documented for all LPS mutants in response to challenges with cephalothin and EGTA, as well. Bbs-12 exhibited slight increases in susceptibility to tetracycline and sulphamethoxazole.

A slight decrease in sensitivity to rifampicin and erythromycin and an increased sensitivity to trimethoprim was consistent for Bbs-5, Bbs-6, Bbs-9, and Bbs-12 when compared with the parental strain, 110H-n.

D. DISCUSSION

Of the *Bordetellae, B. bronchiseptica* is the most similar to other widely studied Gram negatives organisms such as *Pseudomonas* and *Salmonella* species. It does not require special growth conditions, is found widely in the environment and although in possession of many of the virulence factors of *B. pertussis,* usually poses a human health problem only under opportunistic circumstances (16,28,44). As well, where the clinical pathology of other *Bordetellae* species is non-invasive, *B. bronchiseptica* has been isolated from sites other than the respiratory tract (7,44). The source of the drastic difference in clinical presentation, given the high degree of homology between the two species, is not clearly understood.

As a major constituent of the outer membrane the LPS plays a primary role in the interactions between bacteria and host. Studies with *Salmonella typhimurium* LPS truncations have established the importance of LPS structure to bacterial virulence (22,35). The differences in LPS structure between *B. bronchiseptica* and other *Bordetellae,* therefore, represent a reasonable point at which to begin investigations into the structural basis of the pathological variation between the *Bordetellae.*

The LPS of *B. bronchiseptica* is both similar and dissimilar to that of *B. pertussis.* The core is homologous between the two species however, *B. bronchiseptica* possesses a "full" O-unit while *B. pertussis* LOS contains only a very truncated O-chain of three polysaccharides (8,10). The relevance of the extended O-unit to the *in vivo* conformation and activity of *B. bronchiseptica* LPS is not known. To examine the consequences of alterations in the LPS structure on *B. bronchiseptica* pathogenicity we created a series of Tn*5* insertion mutants with truncations in the O-unit and core of their LPS.

After negative screening of mutants using an O-unit specific mAb, we confirmed the phenotypic alterations by SDS-PAGE and the genotypic alteration by Southern blot. Only one Tn*5* insert was present in each mutant and no notable homology was apparent between any 2 genes of the 6 mutants tested. Although all mutants grew similarly well in broth, extraction of LPS from those strains with the greatest truncations, Bbs-6 and Bbs-12, was difficult (Table IV.2). Indeed as the virulent phenotypes produce LPS bands of a slightly higher relative mobility, and the published variants of *B. bronchiseptica* have included only virulent LPSA, LPSC and avirulent LPSB, LPSD phenotypes, Bbs-12 is possibly the roughest LPS phenotype documented from *B. bronchiseptica* to date.

The initial interaction between host and bacteria occurs though consistent, distinct, exposed sites on the bacterial outer membrane (17,26,37). Thus, we assayed the LPS mutants for differences caused in the exposed LPS and cellular epitopes by LPS alterations with the aid of a number of unique and specific cross-reactive monoclonal antibodies from several *Bordetella* species (2,6,11,19,23).

Several deviations in reactivity were notable from what was expected based on the SDS-PAGE profile of the mutant LPS. In several western blots of isolated mutant LPS reacted with D13B11 there was a unique presentation of the O-unit that had not been previously observed in any of our natural isolates. Distinct amounts of O-chain material was evident in D13B11-reacted western blots of Bbs-8, Bbs-9, Bbs-12, Bbs-14, Bbs- and Bbs-25 but absent from silver stained SDS-PAGE gels (Figure IV.1, Table IV.1). This apparent incongruity is most likely the consequence of a decrease in O-chain concentration in the LPS structure but it could also be due to a change in structure greatly affecting the ability of the silver stain to properly recognize the carbohydrate groups. The other *B. parapertussis* O-chain mAb, A7D12, reacted only with Bbs-8 and Bbs-9. The mutants were further differentiated based on exposed regions that are shared between *B. bronchiseptica* and *B. pertussis* as evidenced by the binding of the *B. pertussis* monoclonal antibodies (changes in epitope exposure). Whereas 1H2 reacted to the core region LPSB band only, BL-2 reacted with some additional higher molecular weight bands on Bbs-2, Bbs-8 and Bbs-9; and 14A8 reacted with similar high molecular weight bands on Bbs-1, as well (Figure IV.1B). The western blot data indicates a substantial change has occurred in those regions of the the *B. bronchiseptica* mutant LPS which are exposed to the extracellular environment and therefore available to bind with the various monoclonal antibodies. Exposure of both core (*B. pertussis*: 1H2, BL-2) and O-unit (*B. parapertussis*: D13B11, A7D12) epitopes common to *B. bronchiseptica* and other *Bordetellae* have been altered, indicating a notable change in the overall LPS and outer membrane structure.

The exposure of LPS as presented on the whole cell was determined using colony blots and was comparable to the reactivity of isolated LPS (Table IV.1).

Whole cell ELISAs using D13B11, 1H2, BL-2, 14A8, and A7D12 confirmed the reactivities as detailed in the Western blot data (data not shown).

In an effort to establish the in vivo relevance of these LPS alterations on bacterial survival the complement-mediated bactericidal activity of D13B11 against representatives of the five different mutant phenotypes was analysed. The bactericidal data from analysis of control strains by the previous broth/dilution assays carried out in this laboratory (Peppler et al, manuscript in preparation) was in disagreement with the results obtained with control strain, 110H+, with the agar immunodiffusion protocol used in this study. This is possibly another consequence of the differences in epitope exposure which would result in presenting the bacteria in a solid media such as agar as opposed to broth. It may be that the particular epitope required for bactericidal activity is not available to the mAb when the bacteria is presented on a solid media such as agar.

Truncation of the LPS appears to increase the bactericidal activity of D13B11 in all cases but that of Bbs-6, which by all assays used, is totally deficient of O-chain material (Figure IV.3). Bbs-12, although possessed of a "deeper" core truncation by SDS-PAGE, did exhibit some slight amount of heterogeneity in its possession of higher molecular weight material which reacted with D13B11 on Western blots. Although no D13B11-reactive O-material could be detected on Western blots of isolated Bbs-5 the clear bactericidal activity of this monoclonal antibody suggests that some O-sugars are present albeit in very low quantity. That even such a low amount of D13B11 binding could lead to bacterial lysis indicates the possible presence of another serum sensitivity factor in the outer membrane; possibly the Brk proteins recently discovered in *B. pertussis* and *B. bronchiseptica*.

To extend these findings to an assay of greater *in vivo* relevance the effect of killing by whole human sera was next investigated. Unlike the other *Bordetellae*, *B. bronchiseptica* has been known to cause invasive disease therefore the evasion of the bactericidal mechanisms of human sera is of direct clinical relevance (7,44). Whole human sera has the potential to kill bacteria through a number of systems, several of which are directly affected by the LPS structure. LPS has been documented to activate complement-mediated bacterial lysis by the alternate and terminal complement pathways as well as an antibody-independant classical pathway in addition to the antibody-dependant classical pathway utilized in bactericidal assays (7,14,15,24,29). The antibodies present in normal sera are far more diverse than the mAb utilized in the bactericidal assay increasing the chance that an antibody will be capable of inducing all the steps required for bacterial lysis; activation of the classical complement pathway, formation of the membrane attack complex and final lysis of the cell. The virulent *B. bronchiseptica* strain, 110H+, was as resistant as the avirulent strain, Bbs110H-n. All mutants were greatly increased in their sensitivity to complement-mediated killing by normal human serum (Figure IV.4). The mutants with the most truncated LPS; Bbs-6, Bbs-12 and Bbs-25, were slightly more sensitive than the other mutants.

In previous studies, the LPS O-chain of *B. bronchiseptica* was implicated as a barrier to the outer membrane attachment of the activated terminal complement complex of the classical pathway (7). Where viable cell numbers of a "rough" *B. bronchiseptica* strain (lacking O-unit) decreased by 99.99% upon exposure to porcine hyperimmune serum, no decrease in viable cell numbers was observed in identically treated smooth (complete O-unit) *B. bronchiseptica* strains. These experiments confirm this role for the O-unit in serum resistance and indicate an additional role in serum resistance for a site associated with the core.

Antibiotic sensitivity assays indicated some consistent, minor differences amongst the mutants in comparison with the parental strain, 110H-n. Bbs-12, whose LPS had the greatest truncation, was the most changed of the mutants in its susceptibility to membrane-active agents. Overall, the truncation of the LPS O-unit did not result in large changes in antibiotic sensitivity. The LPS mutants can not be considered anything other than resistant to all of the agents assayed. Thus, the presence of the O-unit, alone, does not explain the dramatic lack of sensitivity to most antibiotics which is uniquely characteristic of *B. bronchiseptica* amongst the *Bordetellae* species.

The data presented in this study indicates that we have created a number of mutants which have LPS that varies significantly in structure and exposure from the wildtype strain. These alterations have resulted in increased susceptibility to killing by human serum but have had only minor effect on the sensitivity of the strains to a wide number of antibiotic and membrane-active agents. Studies on the biochemical and genetic source of the individual mutations should help define the structural basis for these changes and provide a basis for further analysis of the *in* *vivo* disruptions mediated by alteration of *B. bronchiseptica* LPS (i.e. changes in pyrogenicity, mitogenicity, invasion and virulence in the mouse model).

TABLE IV.1: Phenotypic characterization of control and mutant strains of *Bordetella bronchiseptica*.

LPS PHENOTYPE	STRAIN	SDS-PAGE[a] PROFILE	COLONY BLOT[b]	WESTERN BLOT ANALYSIS[b]				
			D13B11 (Bpp mAb) (O-chain)	D13B11 (Bpp mAb) (O-chain)	A7D12[c] (Bpp mAb) (O-chain)	1H2 (Bp mAb) (LOSA)	BL-2 (Bp mAb) (LOSA)	14A8 (Bp mAb) (LOSA)
Control LPS with complete O-chain								
	Bbs 110H-n	B,D	++	++	+	+	+	++
	Bbs110H+	A,C	++	ND[d]	++	+	++	++
1) LPS with B,D and altered O-chain								
	Bbs-14	B,D	+/-	4 BANDS O-CHAIN	-	+	+	+
	Bbs-15	B,D	+/-	HIGH, LIGHT, O-CHAIN	-	+	+	+
	Bbs-16	B,D	+/-	4 BANDS O-CHAIN	-	+	+	+
	Bbs-20	B,D	+/-	4 BANDS O-CHAIN	-	+	+	+
	Bbs-23	B,D	+/-	4 BANDS O-CHAIN	-	+	+	+
	Bbs-25	B,D	+/-	3 BANDS O-CHAIN	-	+	+	+

TABLE IV.1: (continued)

LPS PHENOTYPE	STRAIN	SDS-PAGE^{a} PROFILE	COLONY BLOT^{b}	WESTERN BLOT ANALYSIS^{b}				
			D13B11 (Bpp mAb) (O-chain)	D13B11 (Bpp mAb) (O-chain)	A7D12^{c} (Bpp mAb) (O-chain)	1H2 (Bp mAb) (LOSA)	BL-2 (Bp mAb) (LOSA)	14A8 (Bp mAb) (LOSA)
2a) LPS with B,D and No O-chain								
	Bbs-1	B,D	-	-	-	+	+	+ O-CHAIN
	Bbs-3	B,D	-	-	-	+	+	+
	Bbs-4	B,D	-	-	-	+	+	+
	Bbs-7	B,D	-	-	-	+	+	+
	Bbs-10	B,D	-	-	-	+	+	+
	Bbs-17	B,D	-	-	-	+	+	+
	Bbs-18	B,D	-	-	-	+	+	+
	Bbs-19	B,D	+/-	-	-	+	+	+
	Bbs-22	B,D	-	-	-	+	+	+
	Bbs-24	B,D	-	-	-	+	+	+
	Bbs-26	B,D	-	-	-	+	+	+

TABLE IV.1: (continued)

LPS PHENOTYPE	STRAIN	SDS-PAGE[a] PROFILE	COLONY BLOT[b]	WESTERN BLOT ANALYSIS[b]				
			D13B11 (Bpp mAb) (O-chain)	D13B11 (Bpp mAb) (O-chain)	A7D12[c] (Bpp mAb) (O-chain)	1H2 (Bp mAb) (LOSA)	BL-2 (Bp mAb) (LOSA)	14A8 (Bp mAb) (LOSA)
2b) LPS with B, very little D and No O-chain								
	Bbs-2	B,D	-	-	-	+	+ O-CHAIN	+ O-CHAIN
	Bbs-5	B,D	-	-	-	+	+	+
3) LPS with B and O-chain								
	Bbs-8	B	+/-	FULL O-CHAIN	+	+	+ O-CHAIN	+ O-CHAIN
	Bbs-9	B	+/-	FULL O-CHAIN	+/-	+	+ O-CHAIN	+ O-CHAIN
4) LPS with D, only								
	Bbs-6	D	-	-	-	-	-	-
	Bbs-11	D	-	-	-	-	-	-
	Bbs-13	D	-	-	-	-	-	-
	Bbs-21	D	-	-	-	-	-	-
5) LPS with a band lower than D (D-)								
	Bbs-12	D-	+/-	LOW, LIGHT, O-CHAIN	-	-	-	-

TABLE IV.1: (continued)

[a]Wildtype avirulent parental strain 110H-n has an LPS profile which presents on SDS-PAGE with an O-chain moiety and two low molecular weight bands denoted B and D. This is in comparison to the virulent 110H+ strain which presents with two slightly higher molecular weight bands denoted A and C.

[b] ++ : very strong reaction with strain;

+ : strong reaction with strain;

+/-: weak reaction with strain;

- : no reaction with strain.

[c] Data not shown in Figure IV.1.

[d] Not determined.

TABLE IV.2: Growth and LPS Extraction of *Bordetella bronchiseptica* LPS Mutant and Control Strains

STRAIN	PHENOTYPE (SDS-PAGE, WESTERN BLOT)	BACTERIAL YIELD in g (WET WEIGHT / L CULTURE)	LPS YIELD in g (DRY WEIGHT / L CULTURE)	% WEIGHT IN COMPARISON WITH 110H-n (parent strain)
Bordetella bronchiseptica				
110H-n	O+,LPSB, LPSD	16.49	0.034	100.0 %
110H+	O+,LPSA, LPSC	15.91	0.038	111.8 %
Group 1				
Bbs- 14	LPSB,LPSD	18.00	0.051	150.0 %
Bbs- 25	O+/-,LPSB,LPSD	16.03	0.021	61.76 %
Group 2				
Bbs- 5	LPSB	15.90	0.035	102.9 %
Group 3				
Bbs- 9	O+/-,LPSB	18.66	0.043	126.5 %
Group 4				
Bbs- 6	LPSD	16.01	0.008	23.35 %
Group 5				
Bbs- 12	LPSD-	15.80	0.003	8.824 %

TABLE IV.3: Alterations in Antibiotic Sensitivity Of *Bordetella bronchiseptica* LPS Mutant Strains

BACTERIAL STRAINS	ANTIBIOTIC SENSITIVITY (MIC in μg/ml)										
	Notable Alterations in Sensitivity[a]				Slight Alterations in Sensitivity[b]						
	Increase in Sensitivity	Tn5-Related Decrease in Sensitivity			Increase in Sensitivity					Decrease in Sensitivity	
	Ticaricillin	Amikacin	Kanamycin	Streptomycin	Cephalothin	EGTA	Sulpha-methoxazole	Tetracycline	Trimethoprim	Erythromycin	Rifampicin
Control											
110H-n	3200	8.00	16.0	312	1280	12.5 mM	12.5	25.0	80.0	160	32.0
Mutants											
Group 1											
Bbs-14	1600	128	>128	1250	640	6.25 mM	12.5	25.0	80.0	160	32.0
Bbs-25	800	128	>128	2500	640	6.25 mM	12.5	25.0	80.0	160	32.0
Group 2											
Bbs-5	1600	64.0	>128	2500	640	12.5 mM	12.5	25.0	40.0	320	64.0
Group 3											
Bbs-9	800	128	>128	2500	640	6.25 mM	12.5	25.0	40.0	320	64.0
Group 4											
Bbs-6	800	64.0	>128	1250	640	6.25 mM	12.5	25.0	40.0	320	64.0
Group 5											
Bbs-12	1600	128	>128	1250	640	6.25 mM	6.25	12.5	40.0	320	48.0

a) "Notable" refers to a difference of at least 2 twofold dilutions in at least one of the mutants when compared with the parental strain in 2 separate assays (each strain tested in duplicate/assay).

b) "Slight " refers to a difference of less than 2 twofold dilutions in at least one of the mutants when compared with the parental strain in 2 separate assays (each strain tested in duplicate/assay).

FIGURE IV.1

A) Silver-stained SDS-PAGE profile of lipopolysaccharide from proteinase K-treated Laemmli digests of *B. bronchiseptica* parental strain, 110H-n, and Tn*5*-insert LPS mutants: Group1▲▼- Bbs-14, Bbs-25. Group2☐ - Bbs-5. Group3◇ - Bbs-9. Group4● - Bbs-6. Group5○ - Bbs-12.

B) Western immunoblot assay of reactivity of LPS-specific monoclonal antibodies with SDS-PAGE of proteinase K - treated Laemmli digests of *B. bronchiseptica* parental strain, 110H-n, its virulent variant 110H+ and Tn*5*-insert LPS mutants: Group1▲▼- Bbs-14, Bbs-25. Group2☐ - Bbs-5. Group3◇ - Bbs-9. Group4● - Bbs-6. Group5○ - Bbs-12.

a) Reactivity of *B. parapertussis* O-chain-specific monoclonal antibody D13B11 with SDS-PAGE separated PK-Laemmli digests of *B. bronchiseptica* standard and mutant strains.

b) Reactivity of *B. pertussis* LOSA-specific monoclonal antibody BL-2 with SDS-PAGE separated PK-Laemmli digests of *B. bronchiseptica* standard and mutant strains.

c) Reactivity of *B. pertussis* LOSA-specific monoclonal antibody 1H2 with SDS-PAGE separated PK-Laemmli digests of *B. bronchiseptica* standard and mutant strains.

d) Reactivity of *B. pertussis* LOSA-specific monoclonal antibody 14A8 with SDS-PAGE separated PK-Laemmli digests of *B. bronchiseptica* standard and mutant strains.

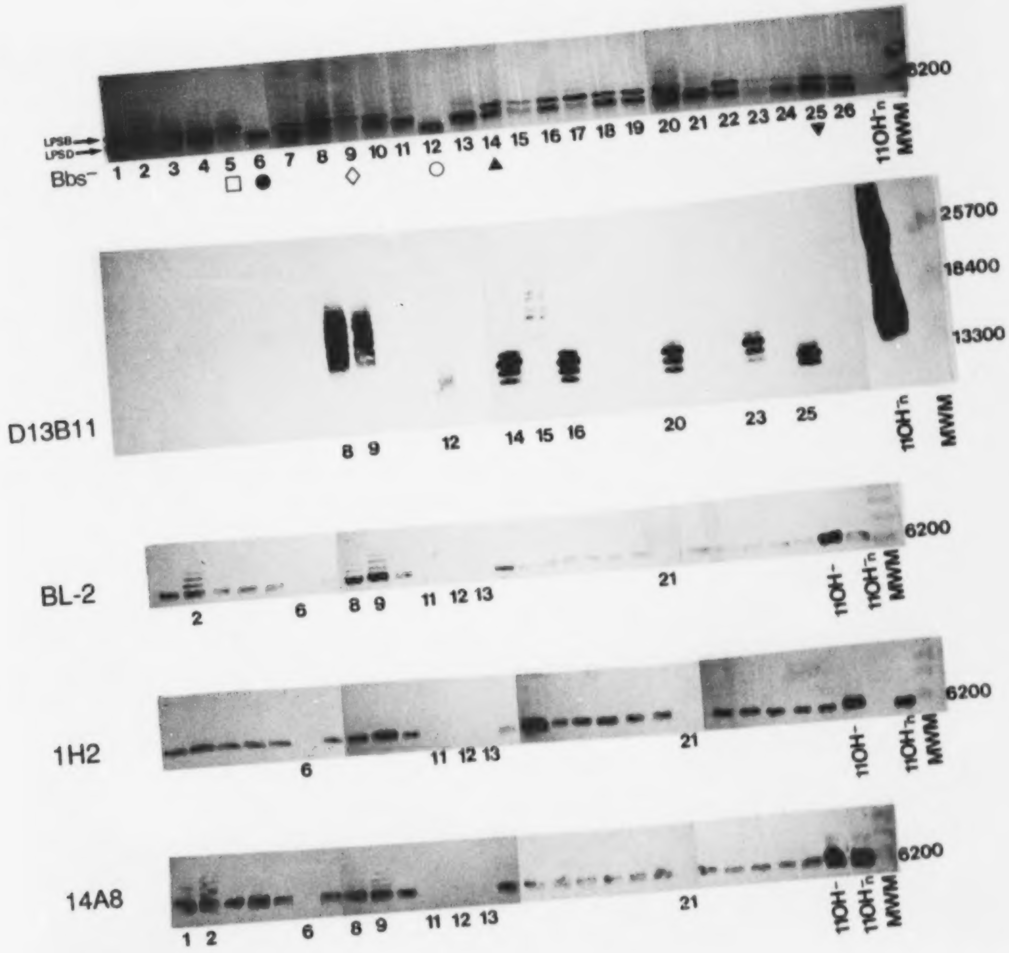

6200
LPSB
LPSD
Bbs⁻ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
□ ● ◇ ○ ▲ ▼
11OH⁻n
MWM
25700
18400
13300
D13B11
8 9 12 14 15 16 20 23 25
11OH⁻n
MWM
BL-2
6200
2 6 8 9 11 12 13 21
11OH⁻
11OH⁻n
MWM
1H2
6200
6 11 12 13 21
11OH⁻
11OH⁻n
MWM
14A8
6200
1 2 6 8 9 11 12 13 21
11OH⁻
11OH⁻n
MWM

FIGURE IV.2

Southern blot analysis of DNA extracted from *B. bronchiseptica* standard and Tn*5*-mutant strains. Autoradiographs of restriction digests probed with a ^{32}P-labeled Tn*5* probe.

a) DNA restricted with *Bam*H1

b) DNA restricted with *Cla*1

c) DNA restricted with *Eco*R1

d) DNA restricted with *Sal*1

Molecular weight markers are shown as follows (in kb): a) 6.6, b) 4.4, c) 2.3, d) 2.0.

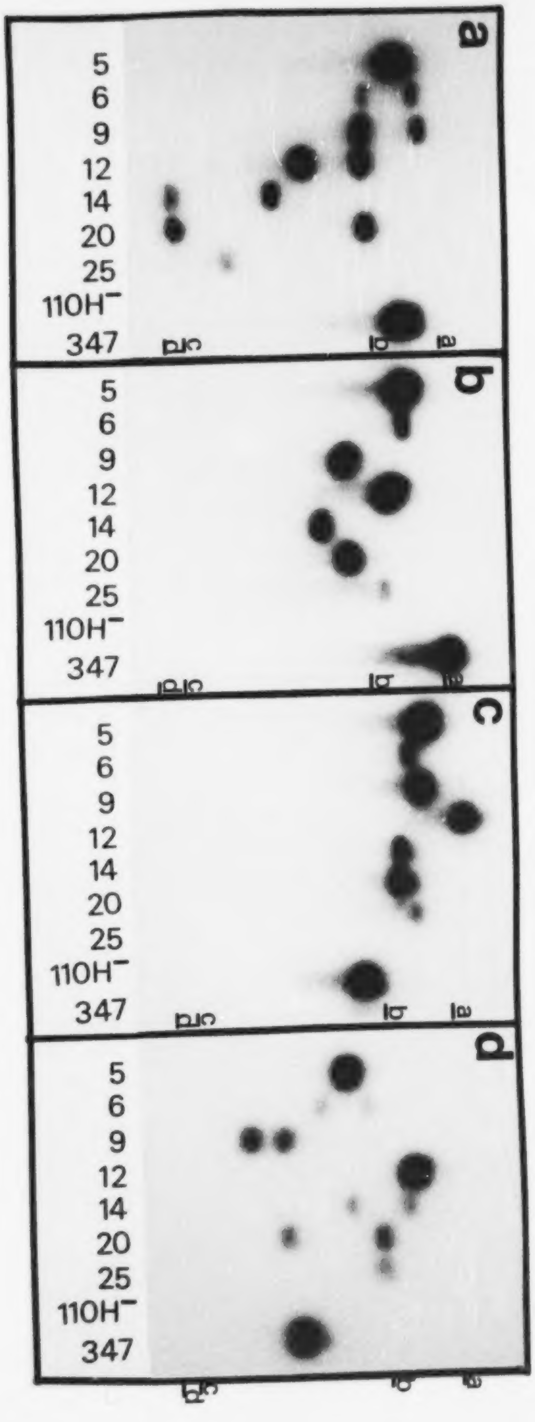

a
b
c
d
5
6
9
12
14
20
25
11OH−
347
a
b
c
d

FIGURE IV.3

Bactericidal activity of *Bordetella parapertussis* O-chain monoclonal antibody, D13B11, against *B. bronchiseptica* Tn*5* mutants with altered LPS. Guinea pig serum was diluted 1:4 to a level where bacterial lysis did not occur as the result of incubation with complement, alone. Antibody was added at an ascites dilution of 1:100. Bars indicate standard deviation amongst results of 4 independant experiments done in pentuplicate; that is each sample was tested 5 times for each of 4 independent experiments. $p<.001$ by student t-test for 110H-n vs. all mutant strains save Bbs-6 and 110H+ ($p = 1.000$).

Bactericidal activity of *Bordetella parapertussis* O-chain mAb D13B11 against *Bordetella bronchiseptica* mutants with altered LPS.

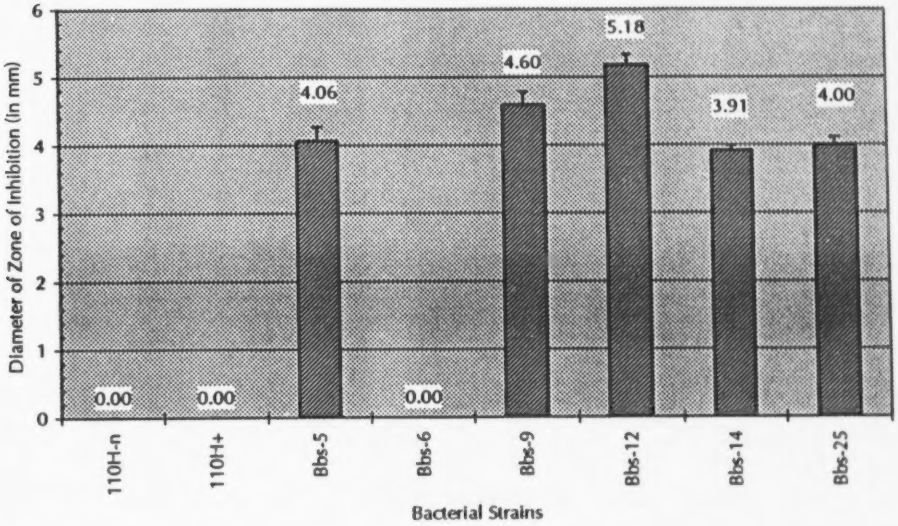
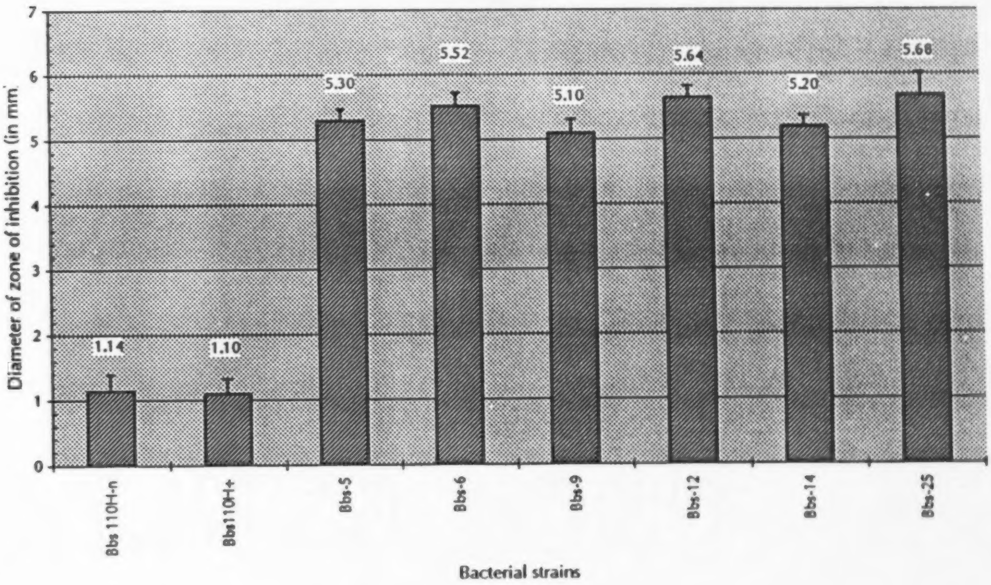


FIGURE IV.4

Sensitivity of *Bordetella bronchiseptica* Tn*5* mutants with altered LPS to pooled normal human sera. Bars indicate standard deviation amongst results of 4 independant experiments done in pentuplicate; that is each sample was tested 5 times for each of 4 independent experiments. p<.001 by student t-test for 110H-n vs. all mutant strains.



Sensitivity of *Bordetella bronchiseptica* control and mutant strains to killing by normal human serum.

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CHAPTER VI

Summary And Conclusions

A. SUMMARY

The mechanics and functioning of the Gram-negative outer membrane have repeatedly proven sensitive to alterations in the structure of endotoxin, the principal component of the extracellular leaflet of the outer membrane. In the complex, multifactorial process of *Bordetellae* pathogenicity, the clinical contribution of each factor should ideally be assessed in the associations and interactions which would normally occur in the whole cell. The whole is often equal to more than the mere sum of its parts. Although a number of previous studies have characterised the properties of the isolated endotoxins of the *Bordetellae*, this is the first study to examine the properties of this molecule as a component of the outer membrane; in the relationships and molecular associations of the whole cell (1,2,4,10,20). The only previously published examinations of LOS whole cell associations investigated the relationship between isolated *B. pertussis* lipooligosaccharide (LOS) and pertussis toxin, another important outer membrane-associated molecule, which established certain synergistic associations in addition to a binding site for enteric LPS on the S2 subunit of PT (8,9,13,16).

In an effort to clarify the role of *Bordetella* endotoxin I undertook investigations which involved the construction, characterisation, and comparison of *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica* mutant strains with genetically altered endotoxin.

B. BIOLOGICAL CONSEQUENCES OF ALTERATIONS IN *BORDETELLA* ENDOTOXIN

i) Comparison of *Bordetellae* sensitivity to complement-mediated bactericidal activity of the D13B11 monoclonal antibody.

A large number of mutants were screened for either reactivity with an LOSB-reactive *B. pertussis* mAb or lack of reactivity with a mAb specific to the O-unit of *B. parapertussis* and *B. bronchiseptica.* In hindsight the negative selection was the more productive of the two methods. Screening of *B. pertussis* mutants for binding to the LOSB-specific mAb, BL-8, excluded the selection of "rougher" mutants or strains with an LOS that was more truncated than LOSB. It would be of interest to examine the consequences of more severe truncation of the LOS core and the extent to which this truncation could occur before the LOS became too unstable and the mutation became lethal. Of the mutant strains confirmed to contain altered endotoxin, a more workable number of mutants was selected which each represented a distinct phenotypic alteration from the parental endotoxin phenotype. The selected mutants were then examined for alterations in outer membrane function and structure.

B. parapertussis and *B. bronchiseptica* were both assayed for sensitivity to complement-mediated lysis by the D13B11 mAb. This monoclonal antibody was raised against *B. parapertussis* but as *B. bronchiseptica* has a chemically identical O-unit the mAb is reactive against *B. bronchiseptica* as well (5). Results for each species differed substantially. Strains of *B. parapertussis* with LPS alterations were considerably less sensitive to the bactericidal activity of D13B11 than strains with native or near native LPS phenotypes. This is expected as the D13B11 mAb is reactive with an epitope found on the O-unit; an epitope which may be missing or altered due to the structural modification of the O-unit observed in the mutant strains. In comparison, LPS mutant strains of *B. bronchiseptica,* were notably more sensitive to D13B11 driven complement-mediated lysis than control or parental phenotypes. In *B. bronchiseptica* the LPS truncations served to make the epitope more, rather than less, accessible to the mAb. With the exception of Bbs-5, only the most drastically truncated mutant with no evidence of higher molecular weight material, Bbs-6, was insensitive to binding and lysis. The *B. bronchiseptica* LPS mutants in contrast with the *B. parapertussis* mutants were sensitive to even small amounts of D13B11 (undetectable by western blot as in Bbs-5). Even a small number of O-containing LPS molecules allows enough D13B11 binding to enable a stable complement attack complex to form and lyse the bacteria. Although the isolated structure of the O-units of *B. parapertussis* and *B. bronchiseptica* may be identical, it is evident from the differences in reactivity to the D13B11 mAb that some additional factor is involved.

It is notable that the Brk proteins which have been proven to mediate resistance to antibody-dependant complement killing of *B. pertussis* are expressed in *B. pertussis* and *B. bronchiseptica* but not in *B. parapertussis* (6, personal communication). Thus, it could be an LPS-dependant change in Brk protein quantity or exposure which is responsible for the increased bactericidal activity of D13B11 in B. *bronchiseptica*. It is also possible that differences in other endotoxin-associated proteins or cations could be the cause of this variation. The nature of this variation, however, remains a subject of study.

ii) Comparison of *Bordetellae* sensitivity to normal pooled human sera.

The sensitivity of endotoxin mutants of the three *Bordetella* species to human serum was less polar. All mutants of *B. parapertussis* and *B. bronchiseptica* were notably more sensitive than parental strains. Past studies indicate that the LPS O-unit of *B. bronchiseptica* and *B. parapertussis* act as a physical barrier to attachment of the membrane lytic complex engendered by the activity of human serum components such as complement (3). The differences in serum sensitivity of *B. bronchiseptica* LPS mutants were consistently, about twice those of *B. parapertussis* mutants indicating a greater role for the LPS O-unit in barrier protection of the *B. bronchiseptica* cell from lysis by the components of whole human serum. This is understandable from a clinical point of view in that *B. bronchiseptica* is the only *Bordetella* species under study known to cause invasive disease (12,19,22). Thus, of the three species only *B. bronchiseptica* is likely to interact closely with large amounts of human serum in addition to the serum defence systems in the mucous of the upper respiratory tract where primary infection of all three *Bordetella* species typically occurs (12,21).

In contrast to the studies of *B. pertussis*, no variation in serum sensitivity was observed between virulent and avirulent phenotypes of *B. bronchiseptica* (48). As well, *B. pertussis* LOS mutants differed from the other *Bordetella* species in the consistency and degree of their sensitivity. As *B. pertussis* LOS normally lacks an extensive O-unit the structural alterations to the endotoxin were less drastic than in the other *Bordetellae* mutants. The lack of a protective O-unit barrier is reflected in the consistent higher sensitivity of all *B. pertussis* strains in comparison with the other *Bordetellae*. Notable differences between LOS mutant and control strains were consistent with a given serum sample, however, the increased variation in strain sensitivity between serum batches indicates that individual immune responses directed against *B. pertussis* are more pronounced and variable than those directed against the other *Bordetellae*. Overall, alterations in endotoxin structure resulted in notable modifications of bacterial sensitivity to lysis by the components of normal human serum.

iii) Comparison of *Bordetellae* sensitivity to antibiotics and membrane-active agents.

The importance of endotoxin structure to the structure and function of the outer membrane, notably the resistance of the outer membrane to penetration by various hydrophobic and hydrophilic antibiotics, has been documented in Gram-negative species such as *E. coli*, *S. typhimurium*, and *P. aeruginosa* (7,11,14,15,17,18). We examined the *Bordetellae* endotoxin mutants for alterations in outer membrane barrier/exclusion functions by analysing the sensitivity of the mutant strains to various antibiotics and membrane-active agents. The most notable alterations in antibiotic sensitivity of previous studies with enteric bacteria were a 10 to 50 fold increase in susceptibility of LPS mutants to novobiocin and a 1 to 5 fold decrease in sensitivity of mutant strains to tetracycline (14,15). Both *B. pertussis* and *B. parapertussis* endotoxin mutants exhibited substantial increases in sensitivity to novobiocin (approximately 8 fold for *B. pertussis* and 3-6 fold for *B. parapertussis*). As well, a notable decrease in sensitivity to tetracycline of approximately 3-4 fold was observed in LOS mutants of *B. pertussis*. In addition to changes in these previously documented antibiotics, alterations in the endotoxin of each of these two species resulted in changes in sensitivity to several other, previously unstudied antibiotics.

The increase in sensitivity to hydrophobic antibiotics such as novobiocin is attributed to the development of phospholipid bilayer "patches" in the outer membrane as the result of the endotoxin and protein loss caused by weakening of outer membrane intermolecular interactions (7,11,14,15,17,18). The decrease in sensitivity of hydrophilic antibiotics such as tetracycline is thought to result from the decreased penetration of the outer membrane due to the loss of porin proteins (7,11,14,15,17,18). In both *B. parapertussis* and *B. pertussis* these alterations in antibiotic sensitivity indicate dramatic disruptions in outer membrane structure and function due to the modification of the endotoxin component.

In contrast, *B. bronchiseptica* strains with altered endotoxin exhibited only minor differences in sensitivity to the same variety of antibiotics and membrane-active agents. As with the examination of human serum sensitivity, although the core of isolated *B. bronchiseptica* LPS has been proven to be chemically similar to that of *B. pertussis* the truncation of the O-unit of *B. bronchiseptica* does not result in similar alterations in outer membrane function (5). Overall, these data indicate that there are more differences in the outer membrane function and structure of the endotoxins of *B. pertussis, B. bronchiseptica*, as well as *B. parapertussis* than can be surmised from analysis of the isolated molecules.

D. CONCLUSIONS

The complex, dynamic interactions which define the Gram-negative outer membrane are particularly important in the infection and immunity induced by multifactorial pathogens such as the *Bordetellae.* The true nature of *Bordetellae* pathogenicity requires analysis of virulence factors in their native conformations and associations within the structure of the bacterial cell. The studies presented in this thesis represent the initial investigations into the clinical relevance and in vivo function of *Bordetellae* endotoxin. Further genetic and structural characterisation of the mutants, additional assays of in vivo function as well as introduction of secondary mutations in possible associated molecules should greatly clarify the "true" role of *Bordetellae* endotoxin in outer membrane function and clinical pathogenicity.

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